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THE APPLICATION OF PAPER PARTITION CHROMATOGRAPHY TO THE PRODUCTION OF DIPHTHERIA TOXIN.

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FOR some years past now it has been our custom, at these laboratories, to prepare diphtheria toxoid from two different types of digest broth. Toxoid to be used for the hyperimmunization of horses has been prepared almost exclusively from papain digest broth ever since we abandoned the autolysate of pig's stomach-veal infusion medium owing to the difficulty of obtaining veal during the war years. Our colleague, Mr. A. T. Glenny, found that toxoids from such papain digest broths gave as good a response, when used for the hyperimmunization of horses, as those prepared from the older type of medium, and were much superior to toxoids prepared from tryptic digest broths. The latter were liable to give excessive swellings and, when used without the addition of alum, relatively poor antigenic response on repeated injection into horses.

We continued to make our weak tryptic digest broths (total nitrogen content 3·0 mg./ml.) for the preparation of toxoids to be used for diphtheria prophylactics, particularly for A.P.T., because it was found possible to produce higher and more uniformly potent toxin from this type of medium than from papain digest broth. With the former, using 360 litre batches of broth, titres of 55–75 Lf units per ml. were obtained when our routine sub-strain, CN 77, of the Park Williams No. 8 strain of *C. diphtheriae* was grown for 10 days in layers of 2·0–2·5 cm. deep. On the contrary, growth on papain digest broth was seldom as luxuriant as that obtained with tryptic digest broth, and consequently toxin titres were usually only 40–50 Lf units per ml. Batches of papian digest broth which did give better growth, however, usually gave appreciably better toxin production up to 60–65 Lf units per ml.

An attempt was made to eliminate this variation by altering the iron and maltose content of the medium. The addition of iron (0·05 mg. $Fe^{\cdots}$ per litre) did give increased growth, but in most cases gave the wrong type of growth, with that faint opalescence in the depth of the culture which we knew from experience indicated that the optimal iron concentration had been exceeded. Increasing the concentration of maltose from 0·5 per cent. (w/v) to 0·7 per cent (w/v) or 0·8 per cent (w/v) did seem to increase the amount of growth in its early stages, but toxin production was little or no better at the end of the 10-day growth

period. Neither was the poor toxin production due to the occasional deterioration which occurs when the strain is maintained by weekly subcultivation on Loeffler slopes, for when the strain was taken again from the original dried culture no better results were obtained. Various other sub-strains of P.W.8 were also tried, but there was little or no improvement in toxin production.

The introduction by one of us (A. J. W.) at these laboratories of the paper partition chromatography originated by Consden, Gordon and Martin (1944), led us to investigate the variability of our papain digest broths by this means. We were able to detect distinct variations from batch to batch, and there was a correlation between the chromatographic picture of the digest and the subsequent toxin production from that digest. Subsequently it has been found possible to modify our technique for the preparation of these digests, so that they give much more consistent chromatograms and hence more consistent toxin production.

We have investigated also the changes in the chromatographic picture which occurred in the medium during the growth of the organism on, firstly, papain digest broth, and secondly, a weak casein hydrolysate medium fortified by the addition of glycine and the usual growth factors.

METHODS.

Preparation of culture media.

1. *Papain digest broth.*—This broth was prepared essentially as described by Linggood and Fenton (1947). The modification introduced into the preparation of the broth in order to obtain more uniform chromatograms, and hence more consistent toxin production, was to treat the minced beef at pH 7·0 with a trace of papain for 2–3 days in the cold room (4° C.) prior to the digestion at 60° C. with the comparatively large amounts of papain. This pre-treatment of minced meat with alkali and papain was a technique first introduced at these laboratories by Dr. van Heyningen, and used for the preparation of media for the production of *Clostridium welchii* toxins (van Heyningen, 1948). To prepare a 150-litre batch of medium, 50 lb. of minced beef, 11·5 litres of water and 184 ml. of 10N NaOH were mixed together in a Hobart mixer for 15 minutes. Papain (12·5 gm.) was then added and the mixing continued for another 5 minutes. The mixture was allowed to stand at 4° C. for 2–3 days and then 140 litres of water added, and the whole digested for 2½ hours at 58–62° C. and pH 6·8–7·2, using five half-hourly additions of 30 gm. papain. The mixture was adjusted to pH 5·0–5·5 and then treated as previously described (Linggood and Fenton, 1947). The broth was filled out into bottles of rectangular section so as to give a layer 2 cm. deep. The completed medium had a total nitrogen content of 4·0 mg. per ml. and an amino nitrogen content of 1·1 mg. per ml. On this medium the strain was grown for 10 days, but 12 bottles of both the strains employed were harvested at 5 days so as to give us a quicker indication of toxin production.

2. *Weak casein hydrolysate medium.*—A deferrated acid hydrolysate was prepared in bulk by autoclaving casein (Glaxo Light White Soluble) with hydrochloric acid and water, adjusting to pH 5·5–6·0, treating the product with charcoal, and then deferrating according to the technique described by Mueller and Miller (1941). The medium was prepared, as suggested to us by Mr. L. B. Holt, by diluting out the deferrated casein hydrolysate to a total nitrogen content of 1·0 mg. per ml. and then adding 0·15 per cent (w/v) of aminoacetic acid.

Cystine, growth factors (pimelic acid, nictotinic acid and β-alanine) and the appropriate amount of iron were then added, the medium filled out into bottles so as to give a layer 1 cm. deep, sterilized for 10 minutes at 115° C. and the maltose-$CaCl_2$ solution added as described by Mueller and Miller (1941). Such a medium had a total nitrogen content of 1·3 mg. per ml. and a sodium chloride content of 0·45 per cent (w/v). The organism was grown for 7 days only on this medium.

Inoculum.

In the experiments with papain digest broths two sub-strains of Park Williams No. 8 of *C. diphtheriae* were used. Firstly our routine strain, CN 77, which we knew from experience produced toxoids which were highly antigenic when used for the hyperimmunization of horses, and, secondly, a sub-strain, CN 2000, of the " Toronto " strain of Park Williams No. 8. The latter sub-strain was used exclusively for experiments with the weak casein hydrolysate medium, as it was the only sub-strain at our disposal which would produce high titre toxin (60–80 Lf units/ml.) on this medium.

Toxin estimations.

Lf values were determined by the flocculation method (Ramon, 1922 ; Glenny and Okell, 1924).

Paper partition chromatography.

Single dimensional paper chromatograms were made of both culture media and toxins by the method of Consden, Gordon and Martin (1944). Our earlier chromatograms were run on No. 1 Whatman paper with *n*-butanol as the solvent in the presence of ammonia. With later chromatograms No. 4 Whatman paper with *n*-butanol-acetic acid as the solvent (Partridge, 1948) was used, as this combination gave a more satisfactory separation of the amino acids and peptides. We have also employed " collidine " and phenol as solvents with success.

RESULTS.

1. *Papain digest broth.*

In Fig. 1 we give photographs of the chromatograms obtained when three papain digest broths, made on consecutive weeks, were examined by single dimensional paper partition chromatography on No. 1 Whatman paper using *n*-butanol as solvent in the presence of ammonia. Although the separation of amino acids on these chromatograms is by no means good, it will be seen that the appearance of the chromatogram of BP 311 is quite different from those obtained for BP 309 and BP 313, although these three broths had been produced by identical procedures, the only difference being that the beef used for BP 311 had been comparatively stale. The chromatogram of the latter shows much more intense spots in the positions where the valine-methionine and the phenylalanine-leucine groups come and is typical of what we term, in Table I, a good chromatogram.

From Table I it will be seen that both BP 309 and BP 313 gave poor toxin production, while BP 311 was quite good in this respect. The explanation of the good growth and toxin production with BP 311 seemed to be, therefore, that

the latter digest contained an abundance of certain amino acids, while BP 309 and BP 313, on which growth was comparatively poor, contained very much less of these amino acids.

Consequently we continued to chromatograph our routine papain digests, but as the separation of the amino acids with *n*-butanol as solvent in the presence of ammonia was rather poor, we tried *n*-butanol-acetic acid as solvent and found that the latter gave us much superior chromatograms, and in Fig. 2 and 3 we give photographs of typical examples of such chromatograms, Fig. 2 being an example run on Whatman No. 1 paper, and Fig. 3 on Whatman No. 4 paper.

Chromatograms in which papain digest broths were run out in parallel with a representative selection of amino acids showed that the variation from broth to broth lay principally in the content of the valine-methionine-tryptophane and the phenylalanine-isoleucine-leucine groups. In this connection it is interesting to recall that Mueller and Kapnick (1935) found that the amino acids valine, methionine and leucine were essential for the growth of their strain of P.W. 8.

It will be noted that BP 311 was made from comparatively stale meat, and this, it was thought, might explain the "good" chromatogram. In order to imitate the use of stale meat we tried the effect of treating the beef, prior to digestion, with a trace of papain for 2 days at pH 7·0 in the cold room (4° C.), as described in the previous section. Such treatment always resulted in digests which gave "good" chromatograms and consequently good toxin production (Table I).

An examination of Fig. 1–3 and Table I shows that there is a general correlation between the chromatogram obtained from a broth and the toxin production thereon, particularly with the sub-strain CN 77, providing, of course, that the iron content of the broth is suitable.

TABLE I.

Number.	Chromatogram.	Toxin production.			
		5 days.		10 days.	
		CN 77.	CN 2000.	CN 77.	CN 2000.
BP 307	Poor	31	35	35	35
309	,,	27	41	40	50
311	Good	52	62	68	76
313	Poor	30	—	42	—
316	Rather poor	29	47	36	53
319	Moderate	36	48	52	56
321*	Good	44	58	58	74
322	Moderate	34	55	53	68
323	Rather poor	30	54	47	70
324*	Good	42	64	60	86
328	,,	40	59	57	77
329	Moderate	36	57	62	72
333	Good	48	58	60	72
339*	,,	52	76	66	82
340	Poor	31	51	45	65

* The beef used in these batches was treated at pH 7·0 with a trace of papain for 2 days in the cold room prior to digestion.

It was thought to be of interest to examine by paper chromatography these digest broths before and after growth of the organism. A series of digest broths and the corresponding toxins were examined, therefore, by this means, the results being shown in Fig. 4, where it can be seen quite clearly that there is an increase after the growth of the organism in the quantity of almost every amino acid detectable in the *n*-butanol-acetic acid chromatogram, due presumably to the breakdown of proteoses and polypeptides in the original broth. This observation confirms and amplifies our earlier work that, with papain digest broth, there is always an appreciable increase in the amino nitrogen content of the culture filtrate, as measured by the method of Pope and Stevens (1939), during the growth of the organism.

It can be seen from the photographs that with papain digest broth there is a faint background to the chromatograms, which, we think, is due to the presence in the broth of a considerable amount of proteose and polypeptide for, it must be remembered that the ratio of amino nitrogen to total nitrogen in the original broth is usually about 28 per cent, whereas if there were only amino acids present this ratio would be about 70 per cent. Therefore, in order to obtain " cleaner " chromatograms we tried next using a casein hydrolysate medium in which the protein had been completely broken down into simple amino acids.

2. *Weak casein hydrolysate medium.*

As was anticipated, this medium gave much " cleaner " chromatograms than our papain digest broths, and a study of the pictures obtained before and after the growth of the organism, i.e. on the medium and the corresponding toxin, has revealed several interesting points (Fig. 5, 6 and 7). After growth nearly all the cystine, aspartic acid, glutamic acid and threonine which can be detected by paper chromatography have disappeared from the medium, while the alanine content has shown a marked increase. A decrease in the glycine-serine, valine-methionine and leucine groups can also be detected. These findings are consistent with the fact that there is a drop in the amino nitrogen content of the culture filtrate compared with that of the original medium. The increase in the intensity of the alanine spot may possibly be due to the presence of a transaminase in the organism, for it has been shown (Cohen, 1940 ; Darling, 1945) that such an enzyme can convert, for example, glutamic acid into α-alanine.

It was thought to be of interest to study by paper chromatography the effect of growing the organism on this medium to which had been added, instead of an optimal quantity of iron for toxin production, a range of iron from below to above the optimal concentration. The results are shown in Fig. 8, which is a photograph of a chromatogram run with " collidine " as solvent, and shows that even where growth is minimal owing to the lack of iron the loss of aspartic and glutamic acids is still quite marked. The decrease in the intensity of the valine, methionine and leucine spots can also be seen quite clearly on this chromatogram.

DISCUSSION.

The results obtained above indicate quite clearly that paper partition chromatography is of great value in the routine production of, and research work on, diphtheria toxin. It gives a very simple and quick method of obtaining a qualitative, or even a rough quantitative, measure of the amino-acid constituents of enzymatic digests or acid hydrolysates of proteins and of the products obtained

by the growth of organisms thereon. If our papain digest broths had had to be examined by the more laborious methods of chemical or microbiological analysis, we should still be struggling with the problem of the large variations in toxin production on such culture media. The technique of paper partition chromatography has enabled us at least to control another of the many variables in diphtheria toxin production on complex culture media, such as papain digest broths. In Fig. 9 we give photographs of the chromatograms of one of our weakest tryptic digest broths (total nitrogen 2·3 mg./ml.), as well as two papain digest broths, one BP 313, a poor toxin producer, and BP 324, a good toxin producer. This explains, at a glance, why more consistent toxin production was obtained with the former, for it will be seen that all the amino acids which are believed to be essential for good growth and toxin production are present in appreciable amounts in tryptic digest broth.

The results obtained with the weak casein hydrolysate medium are equally interesting, for the chromatograms show quite clearly that cystine, aspartic acid, glutamic acid and threonine are utilized to an extent that may render them the limiting factor for toxin production. Work is now in progress to investigate the effect of small amounts of these amino acids on toxin production from this medium.

SUMMARY.

Paper partition chromatography has been shown to be of great value for the control of routine production of diphtheria toxin from papain digest broth, and has enabled us to devise a modified digest which will give more consistently high titre toxin. It has been shown also that there is an appreciable increase in the

EXPLANATION OF PLATES.

Figs. 1–6 and 9: Key to amino acids in chromatograms run with *n*-butanol as solvent.—(*a*) cystine; (*b*) basic amino acids, i.e. lysine, arginine and histidine; (*c*) aspartic acid; (*d*) glycine and serine group; (*f*) glutamic acid and threonine group; (*h*) alanine; (*k*) proline; (*l*) tyrosine; (*n*) valine-methionine-tryptophane group; (*o*) phenylalanine; (*p*) leucine and isoleucine group.

Fig. 1.—Chromatograms of three papain digest broths run on No. 1 Whatman paper with *n*-butanol as solvent in the presence of ammonia.

Fig. 2.—Chromatograms of three papain digest broths run on No. 1 Whatman paper with *n*-butanol-acetic acid as solvent.

Fig. 3.—Chromatograms of six papain digest broths run on No. 4 Whatman paper with *n*-butanol-acetic acid as solvent.

Fig. 4.—Chromatograms of ten papain digest broths (B) and their corresponding toxins (T) run on No. 4 Whatman paper with *n*-butanol-acetic acid as solvent.

Fig. 5.—Chromatograms of weak casein hydrolysate medium and the corresponding toxins run on No. 4 Whatman paper in parallel with aspartic and glutamic acids using *n*-butanol-acetic acid as solvent.

Fig. 6.—Chromatograms of weak casein hydrolysate medium and the corresponding toxins run on No. 4 Whatman paper in parallel with various amino acids using *n*-butanol-acetic acid as solvent.

Fig. 7.—Chromatograms of casein hydrolysate medium without and with the addition of aspartic and glutamic acids run in parallel with the corresponding toxins on No. 4 Whatman paper with phenol as solvent.

Fig. 8.—Chromatograms of casein hydrolysate medium and the corresponding toxins prepared on this medium with varying additions of $Fe^{\cdots}$ run on No. 4 Whatman paper using "collidine" as solvent.

Fig. 9.—Chromatograms of tryptic and papain digest broths run on No. 4 Whatman paper with *n*-butanol-acetic acid as solvent.

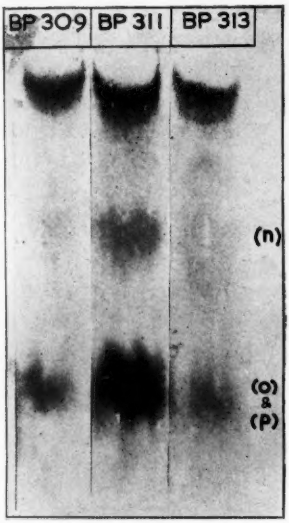

FIG. 1.

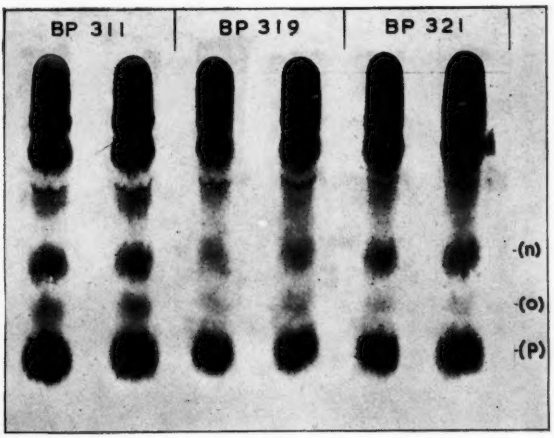

FIG. 2.

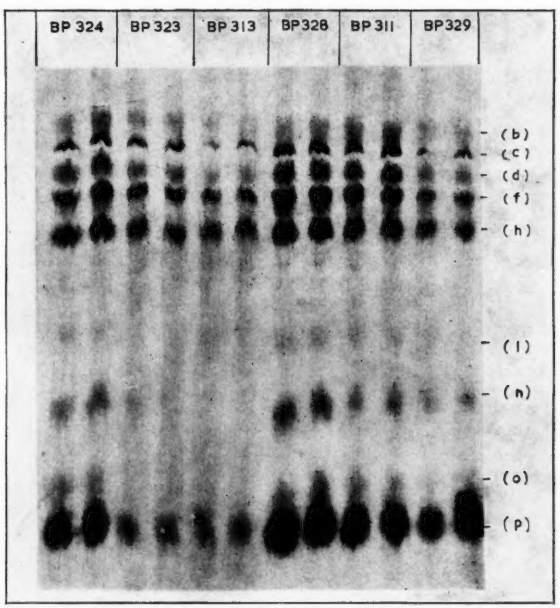

FIG. 3.

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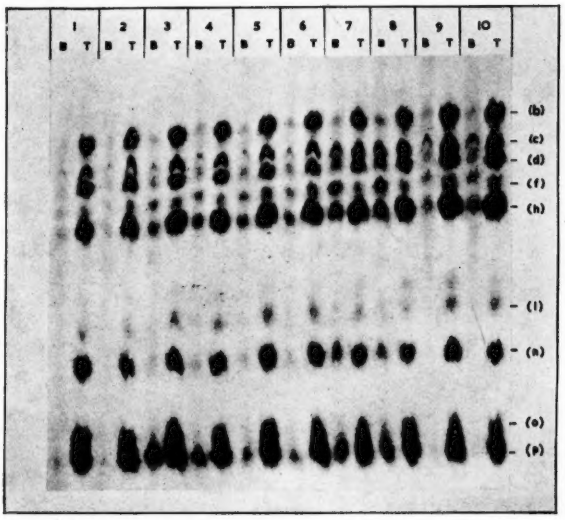
1 2 3 4 5 6 7 8 9 10
B T
(b)
(c)
(d)
(f)
(h)
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(n)
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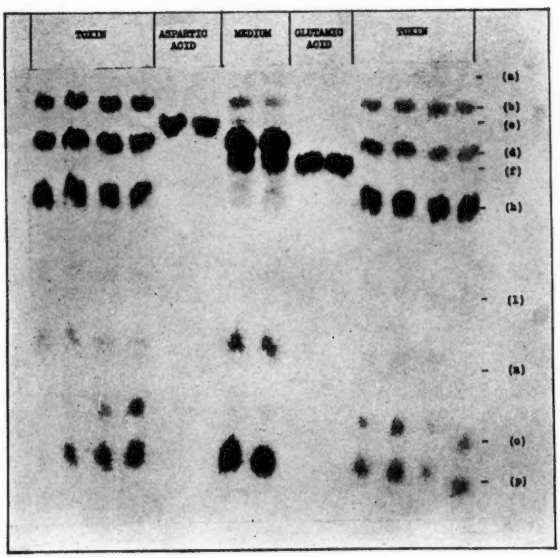
(p)

FIG. 4.


TOKEN
ASPARTIC ACID
MEDIUM
GLUTAMIC ACID
TOKEN
(a)
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(o)
(p)

FIG. 5.

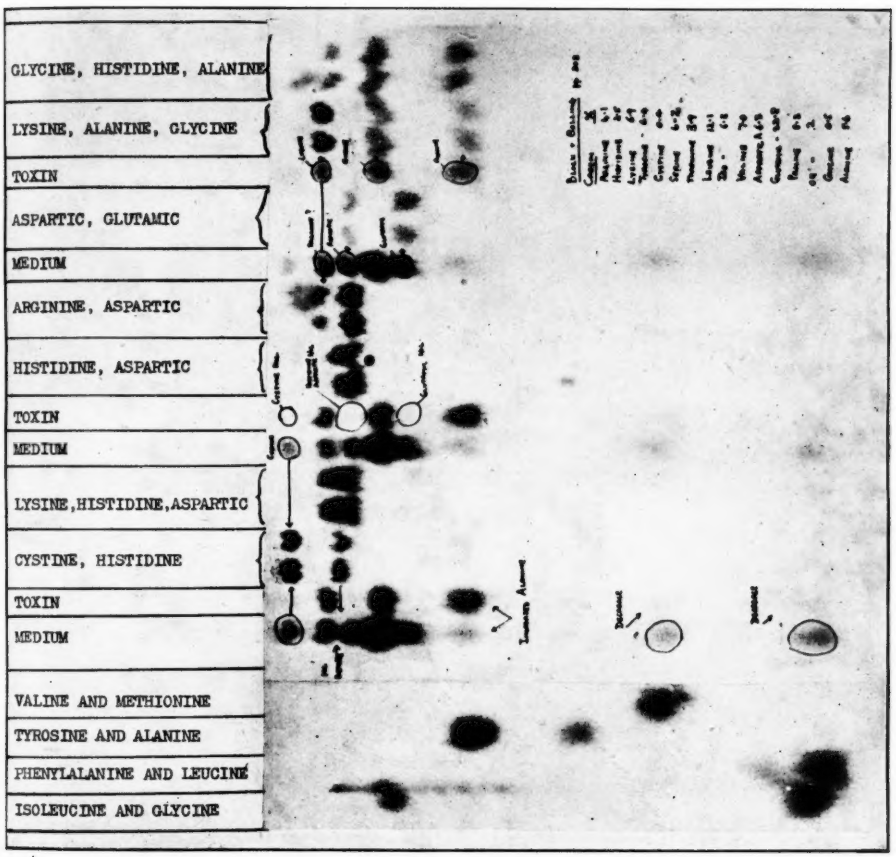
GLYCINE, HISTIDINE, ALANINE
LYSINE, ALANINE, GLYCINE
TOXIN
ASPARTIC, GLUTAMIC
MEDIUM
ARGININE, ASPARTIC
HISTIDINE, ASPARTIC
TOXIN
MEDIUM
LYSINE,HISTIDINE,ASPARTIC
CYSTINE, HISTIDINE
TOXIN
MEDIUM
VALINE AND METHIONINE
TYROSINE AND ALANINE
PHENYLALANINE AND LEUCINE
ISOLEUCINE AND GLYCINE

FIG. 6.

FIG. 7.

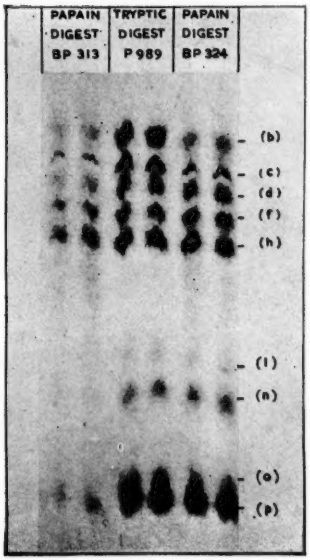

FIG. 9.

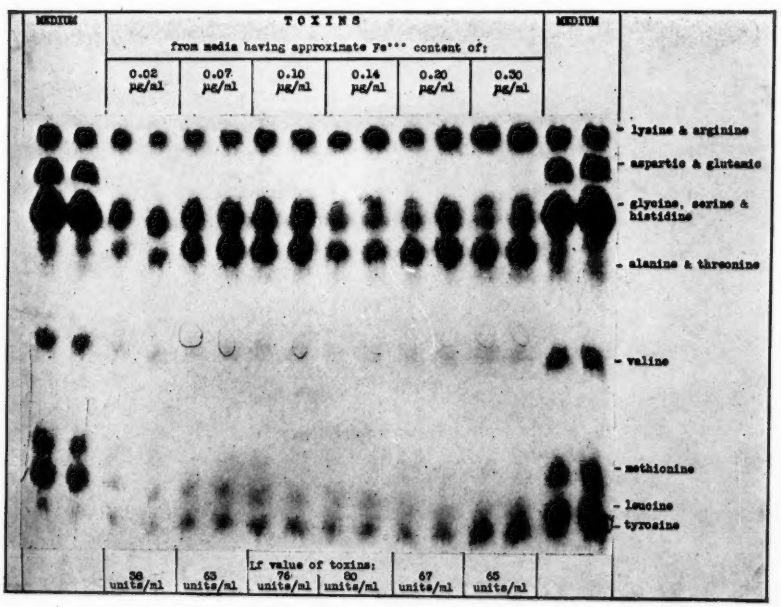

FIG. 8.

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amount of almost every amino acid, detectable with *n*-butanol-acetic acid chromatograms, in the culture filtrate compared with the original medium.

With a weak casein hydrolysate medium, however, nearly all the cystine, aspartic acid, glutamic acid and threonine detectable by paper chromatography are utilized during the growth of the organism, but there is an increase in the quantity of alanine in the culture filtrate as compared with the original casein hydrolysate medium.

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PROTECTION BY STREPTOMYCIN, PENICILLIN AND LICHENIFORMIN AGAINST *C. DIPHTHERIAE* INFECTIONS.

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NEITHER active immunization with diphtheria prophylactics nor treatment with antitoxin results in disappearance of *C. diphtheriae* from carriers, and prolonged stay in hospitals of convalescent patients is frequently necessary before negative swabs are obtained. Even with negative throat swabs *C. diphtheriae* has been isolated from the interior of a removed tonsil (Bagnall and Bain, 1945), and the carrier may become a disseminator of infection. Hence an agent for killing diphtheria bacilli in the body is required.

Although most strains of *C. diphtheriae* are sensitive to penicillin (Fleming, 1929; Abraham, Gardner, Chain, Heatley, Fletcher, Jennings, and Florey, 1941) the results of both local and systemic treatment are disappointing, and in the literature conflicting conclusions concerning the efficacy of penicillin treatment are reported (Paull, Tucker, Hollowày and Nicewonger, 1946; Kocher and Siemsen, 1946; Long, 1946; Borst, 1948; Levy, 1948).

This investigation was undertaken to compare the protective effect in animals against *C. diphtheriae* of penicillin, streptomycin, and of the antibiotic licheniformin described by Callow and Hart (1946), and Callow, Glover, Hart and Hills (1947), with the object of selecting an antibiotic suitable for the treatment of diphtheria carriers and convalescents as well as acute fever patients.

MATERIALS, METHODS AND RESULTS.

Strains.

Since the most common Gravis infections in this country are of Type I most experiments were carried out with a strain of this type, but a few experiments were carried out with other Gravis, Mitis and Intermedius strains. The serological typing and general technique have been described (Hewitt, 1947, 1948).

Antibiotics.

The penicillin used was the crystalline sodium salt containing 90 per cent of penicillin G (Glaxo). Streptomycin (Merck) and licheniformin (Callow and Hart) were used in the form of the hydrochlorides; one of the two batches of licheniformin was rather more toxic to guinea-pigs than the other. Sterile dried preparations of penicillin and streptomycin were dissolved in sterile normal saline. Licheniformin was also dissolved in normal saline and for *in vitro* experiments was autoclaved for 10 minutes at 10 lb. pressure, while for the animal injections the saline solution was used without autoclaving.

In vitro *tests.*

Serial dilutions of the antibiotic in papain digest broth were inoculated with 18-hour broth cultures of the strains investigated.

The minimum concentrations of the three antibiotics inhibiting growth are given in Table I. The amounts given in μg. per ml. are those which prevented the development of any appreciable visible turbidity or pellicle after 48 hours incubation.

TABLE I.—*Minimum Inhibitory Concentrations of Antibiotics against Strains of C. diphtheriae.*

Strain.	Group.	Type.	Inhibitory concentration (μg. per ml.).		
			Penicillin.	Licheniformin.	Streptomycin.
1	Gravis	I	0·1	0·25	2·0
34	Gravis	II	0·1	0·25	2·0
234	Gravis	II	0·25	0·25	1·0

These results are in agreement with previous figures. Eighteen other diphtheria strains (Gravis, Intermedius and Mitis) were inhibited by minimal concentration of penicillin ranging from 0·02 to 0·3 μg. per ml. In the case of licheniformin the inhibitory concentrations of six strains were reported by Callow, Glover, Hart and Hills (1947) to range from 0·1 to 0·8 μg. per ml. Four other diphtheria strains were inhibited by 1–3 μg. per ml. of streptomycin, and Keefer *et al.* (1946) reported streptomycin to be inhibitory to *C. diphtheriae* in concentrations ranging from 0·4 to 4 μg. per ml.

Animal protection experiments.

Guinea-pigs weighing from 250 to 300 g. were used for protection experiments and were infected with saline suspensions of *C. diphtheriae*. 18-hour cultures on Loeffler slopes were examined for purity, washed off and suspended in saline, centrifuged, and the supernatants discarded in order to eliminate pre-formed toxin. The deposit was re-suspended in saline at an opacity corresponding to 1–2 $\times$ 10^9 organisms per ml., and the infecting dose was about 3 $\times$ 10^8 organisms per ml. In one experiment 0·5 ml. was injected subcutaneously, and in all other experiments 0·2 ml. was administered intracutaneously, no difference in result being observed in the two routes of administration. The antibiotics were dissolved in normal saline and the appropriate dose injected subcutaneously. The general procedure in the following experiments unless stated to the contrary was to inject a first dose of the drug fifteen minutes before the infecting dose of organisms, and to give three further doses at two-hour intervals. Animals were weighed daily and autopsies were carried out on all dead animals to exclude the possibility of intercurrent infections.

The results of comparative experiments are given in Table II.

TABLE II.—*Comparative Protection by Different Antibiotics.*

(Infecting dose : strain 234, Gravis Type II, 0·5 ml. subcutaneous.)

Antibiotic.	Doses.	Total dosage (mg.).	Time of death (hours) (S = survived 10 days).
Nil	..	*nil*	26 : 28
Penicillin	4 $\times$ 10 mg.	40	42 : 47
Licheniformin	2 $\times$ 10 mg.	20	27 : 51
Streptomycin	4 $\times$ 10 mg.	40	S : S
Antitoxin	1	500 units.	S : S

The total dosage of licheniformin was reduced to 20 mg. owing to the toxicity of the batch used.

It will be noted that 40 mg. of penicillin and 20 mg. of licheniformin failed to give protection, but 40 mg. of streptomycin completely protected the guinea-pigs, as did diphtheria antitoxin used as a control. Subsequent experiments with lower doses of penicillin (4 $\times$ 2 mg.) and licheniformin (4 $\times$ 2 mg.) indicated no protective effect, but 40 mg. of a less toxic batch of licheniformin prolonged life without giving complete protection. With smaller infecting doses of *C. diphtheriae* protection by licheniformin has been observed (D. G. Evans in a personal communication). Since the streptomycin results appeared promising, further tests were made with different doses as shown in Table III.

It will be seen that streptomycin in 4 doses of 4 mg. or 2 doses of 10 mg. completely protected guinea-pigs against massive infection with *C. diphtheriae*, 4 doses of 2 mg. protected some of the animals, whilst 2 doses of 5 mg., one just before injection and the other six hours later, prolonged life without giving complete protection. Animals protected by streptomycin remained well throughout the experiment, and when discarded ten days later appeared completely normal save for local skin necrosis at the site of injection.

In order to eliminate the possibility that streptomycin protection was manifested only against the two strains tested, 9 pairs of guinea-pigs were infected

with a dose of about 4×10^8 of different strains of *C. diphtheriae*. One animal of each pair received 4 doses of 4 mg. of streptomycin at two-hour intervals. The results are given in Table IV.

TABLE III.—*Protection by Streptomycin against* C. diphtheriae.

(Infecting doses 4×10^8 organisms of strain 1 (Gravis type I) in 0·2 ml. intracutaneously.)

No. of animals in group.	Doses of streptomycin.	Total dosage (mg.).	Time of death (hours).
4	4 × 4 mg.	16	S : S : S : S
2	2 × 10 mg.	20	S : S
4	4 × 2 mg.	8	46 : 46 : S : S
2	2 × 5 mg.	10	70 : 90
2	4 × 1 mg.	4	26 : 45
2	3 × 2 mg.	6	26 : 29
16	*nil*	*nil*	22–27
3	Antitoxin	100 units	S : S : S

TABLE IV.

Strain.	Group.	Type.	Time of death (hours). No streptomycin.	Time of death (hours). Streptomycin (4 × 4 mg.)
393	Gravis	I	33	S
34	Gravis	II	30	S
246	Gravis	III	25	S
8	Intermedius	..	36	S
265	Intermedius	..	36	S
230	Mitis	I	23	S
13	Mitis	II	25	S
424	Mitis	III	35	S
100	Mitis	VII	29	S

In each case 4 doses of 4 mg. of streptomycin gave complete protection.

Development of Resistance.

Penicillin.

The development of a comparatively low degree of resistance followed subculture in serial dilutions of penicillin broth, the culture showing growth in the highest concentration of penicillin being subcultured into a fresh series of dilutions of the antibiotic. Nine diphtheria strains (4 Gravis, 2 Intermedius and 3 Mitis), inhibited in concentrations ranging from 0·002 to 0·2 units per ml., were subcultured 30 times in penicillin broth when the inhibiting range had risen to 0·3 to 0·8 units per ml. With a further nine strains (5 Gravis, 2 Intermedius, 2 Mitis) after 40 subcultures the inhibiting concentrations rose from a range of 0·04 to 0·32 units per ml. to 0·1 to 0·5 units per ml.

Streptomycin.

In contrast to penicillin, resistance to streptomycin of some strains increased rapidly. With one strain (Gravis, Type II) the initial inhibiting dose of 2 μg. per ml. rose to 20 μg. after 7 subcultures and then remained constant for a

further 7 subcultures. With another strain (Gravis, Type I) the initial inhibitory dose of 2 μg. per ml. rose to 20 μg. after 7 subcultures and to 2000 μg. per ml. after 12 subcultures.

Streptomycin resistance is apparently not readily reversed. After nine subcultures in broth resistant strains retained their streptomycin resistance unimpaired. No difference in the fermentation reactions of resistant strains was observed, although some indications of difference in metabolic activities are being further investigated.

In the case of strain 34 (Gravis II) the colonial appearance on tellurite blood agar of the resistant strain was altered from a typically rough Gravis type to that of the smaller smooth Mitis type, but starch was still fermented by the resistant strain, which might therefore be described as "atypical Gravis." The more resistant Gravis I strain showed no change in colonial appearance when resistant and grew vigorously in broth with a surface pellicle, whilst the Gravis II-resistant strain grew rather feebly in broth with development of general turbidity and little surface pellicle—a behaviour which again suggests the development of Mitis-like characteristics.

Toxigenicity of Streptomycin-resistant Strains.

Toxin production of resistant strains in broth cultures was determined as described previously (Hewitt, 1948), and the results are given in Table V. The development of streptomycin resistance had not impaired toxigenicity, and in the case of the streptomycin resistant variant of Strain I the presence of streptomycin in the culture medium (100 μg. per ml.) did not inhibit toxin formation; in fact the amount of toxin produced (3 Lr per ml.) was more than in the absence of the antibiotic.

TABLE V.—*Toxigenicity in Broth of Streptomycin-resistant Gravis Strains.*

Strain.	Type.	Toxigenicity (Lr units per ml.). Original strain.	Resistant strain.
1	I	0·65	1·4
34	II	2·0	0·4

Protection against Resistant Strains.

In order to test whether the resistance to streptomycin developed in broth cultures was manifested also in infections in the body, guinea-pigs were infected with partially and completely resistant strains, with the results given in Table VI.

TABLE VI.—*Protective Effect against Streptomycin-resistant Strains.*

(Original strain 1, Gravis, Type I.)

Number of subcultures in streptomycin broth.	Minimal inhibiting dose of streptomycin in broth (μg. per ml.).	Guinea-pig dose of streptomycin (mg.)	Time of death (hours).
0	2	16	S : S
0	2	0	22 : 23
7	20	16	168 : 192
7	20	0	23 : 27
12	2000	40	27 : 28
12	2000	0	22 : 26

In each case the post-mortem appearances were typical of diphtheria toxaemia.

It will be seen that development of resistance to streptomycin in broth cultures is accompanied by disappearance of the protective effect of streptomycin on infections *in vivo*. The usual protective dosage of 16 mg. (4 doses of 4 mg.) prolonged life, but did not completely protect against a semi-resistant strain, whilst 40 mg. (4 doses of 10 mg.) failed even to prolong life in the case of a completely resistant strain.

DISCUSSION.

Although penicillin is inhibitory to the growth of *C. diphtheriae* in broth in very high dilutions, its protective effect in the body against infection with *C. diphtheriae* is very low. This is in accord with the comparatively disappointing results of penicillin treatment locally or systemically in clearing up human diphtheria infections and carriers, and may be due to the comparatively slow action of penicillin. Licheniformin also has a rather low protective effect.

Streptomycin, on the other hand, is very effective in comparatively low doses, although it is rather less inhibitory in broth. This may be due to the rapidly lethal effect of streptomycin on bacteria reported by Garrod (1948). Four doses of 4 mg. of streptomycin at two-hour intervals gave guinea-pigs complete protection against massive infections with *C. diphtheriae*. Two doses of 10 mg. of streptomycin also gave protection, but smaller doses could not be depended upon to protect completely.

There are, therefore, strong indications that streptomycin should be tested clinically for the treatment of diphtheria patients, carriers and convalescents. In view of the possibility of viable *C. diphtheriae* remaining in the tonsils of persons giving negative swabs, systemic treatment may be necessary, but the use of streptomycin sprays and lozenges for local treatment should be considered. Since streptomycin resistance develops rapidly with some strains of *C. diphtheriae*, the initial dosage should be adequate, since if not effective within a short period the treatment may be useless.

Streptomycin resistance once developed is not readily reversed. The toxigenicity of resistant strains is retained, but one resistant Gravis strain tended to develop some cultural characteristics of the Mitis group whilst retaining its starch fermentability.

SUMMARY.

(1) Streptomycin completely protected guinea-pigs against massive infection with *C. diphtheriae* in a dose of 16 mg. spread over a 6-hour period.

(2) It is suggested that streptomycin might be effective for the treatment of diphtheria carriers and convalescents.

(3) Some strains of *C. diphtheriae* develop streptomycin resistance rapidly. This resistance is manifested not only in broth but in the body. Streptomycin-resistant strains remain toxigenic.

(4) In view of the development of streptomycin resistance, adequate initial dosage is necessary in treatment of diphtheria infections.

(5) Although powerfully inhibitory in broth cultures, penicillin is relatively ineffective in protecting animals against infection with *C. diphtheriae*.

(6) Licheniformin has a comparatively low protective effect against *C. diphtheriae* infections.

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STUDIES ON A FACTOR IN NORMAL ALLANTOIC FLUID INHIBITING INFLUENZA VIRUS HAEMAGGLUTINATION. OCCURRENCE, PHYSICO-CHEMICAL PROPERTIES AND MODE OF ACTION.

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HIRST'S discovery that influenza virus can be adsorbed to and eluted from red cells (Hirst, 1942) and the cells of the respiratory tract (Hirst, 1943) has afforded a possibility, now utilized in various laboratories, of studying the first stages of the virus infection and the conditions relative to the tissue specificity of virus. Hirst's hypothesis that influenza virus is capable of enzymically destroying specific receptors on the red cells has thus obtained further support.

Two separate lines are discernible in these studies. On the one hand there is the study of the modifying effect of different viruses and enzymes, etc., on the adsorptional capacity and agglutinability of the red cell (Burnet, 1945*a*, *b*; 1948; Burnet, McCrea and Stone, 1946; Hirst, 1948), on the other the investigation of a number of tissue substances inhibitive to haemagglutination, especially those which occur in normal serum (Francis, 1947; Burnet, 1948; Burnet, McCrea and Anderson, 1947; Hirst, 1948), and in extract from red cells (Friedewald, Miller and Whatley, 1947; Bovarnick and de Burgh, 1947; de Burgh, Yu, Howe and Bovarnick, 1948).

These inhibitors are considered to be identical or closely analogous to the receptors of the red cells. The working hypothesis is that an inhibitor which produces its effect through combining with the virus particles must be structurally more or less analogous to the receptor substance of the red cells.

Burnet and co-workers (Burnet, 1948) and Hirst (1948), in quite recently published papers, have followed both these lines. They came to the conclusion that the receptors are mucoproteins. The result coincides with a report issued by de Burgh and co-workers (1948), stating that purified preparations of the factor inhibitive to haemagglutination, in extract from red cells, consist largely of polysaccharides.

Stone reported lately (1947) that filtrate of a culture of *Vibrio cholerae* which, earlier (Burnet, McCrea and Stone, 1946; Burnet, McCrea and Anderson, 1947), was found able to destroy the receptors of the red cells and the inhibitor of normal serum, can also render the cells of the allantoic sac of chick embryos insusceptible to influenza virus infection. Consequently Burnet (1948) infers that also the receptors of cells susceptible to infection consist of a mucin, and that the first stage in the infection is the combination of a mucinase in the virus particle with its substrate. After decomposition of the receptor the virus is ready for the next step in the infection.

It has been revealed, however, that a difference may exist between the ability of a virus to render red cells inagglutinable and its capacity to render a chicken embryo insusceptible to subsequent infection (Henle and Henle, 1947; Florman, 1948). This indicates that the reaction between the red cell receptors and virus is no adequate model of the reaction between virus and the receptors of cells susceptible to infection. The ideal would therefore be to employ as test object receptors liberated from cells which are susceptible to virus infection. There is reason to assume that the inhibiting factor, in normal allantoic fluid from chick embryo, which will be described in this paper, fulfils this condition.

Beveridge and Lind (1946) have observed that normal allantoic fluid has an inhibitory effect on mumps virus haemagglutination, and Cunha, Weil, Beard, Taylor, Sharp and Beard (1947) have reported a similar effect with Newcastle disease virus. Preliminary accounts concerning the occurrence and properties of the factor inhibiting influenza virus haemagglutination have been published earlier by the author (Svedmyr, 1947*a*, *b*). In these reports the majority of the results in this paper were briefly put forward.

MATERIALS AND METHODS.

Virus preparations.—The PR8 strain was employed throughout these experiments. Infective fluids were produced by inoculating egg-passaged virus, diluted as a rule 10^{-6}, into the allantoic sac of chicken embryos, 11 days old. After incubation at 35° C. for 48 hours, the eggs were chilled overnight at 4° C. prior to the harvest of allantoic fluid. The fluid was subsequently cleared by centrifugation at 3500 r.p.m. for about 20 minutes, or by filtration through tightly packed cotton-wool. The fluids were stored at 4° C., inocula however at —70° C.

Normal allantoic fluid.—Allantoic fluid from embryos 13 days old was used in the experiments unless otherwise stated. Harvest and clarification as for virus preparations, storage at 4° C.

Haemagglutination tests.—Salk's method (Salk, 1944) somewhat modified by von Magnus (personal communication) was employed. Two twofold serial dilutions of virus suspension were made simultaneously in 0·5 ml. of 0·9 per cent saline. Each dilution in the one series was the geometrical mean of two adjacent dilutions in the other, for instance, the series 1/2, 1/4 and so on, and $1/2\sqrt{2}$, $1/4\sqrt{2}$ and so on. In order to diminish the dilution error the initial concentration was chosen, when feasible, so that the end-point was reached with as few serial dilutions as possible. For the same reason the pipette was changed between each dilution (ungraded pipettes coupled to an automatic, specially devised syringe were used).

To each tube was added 0·5 ml. of 0·25 per cent chicken red cell suspension, and the mixture was allowed to stand at room temperature for 1–1·5 hours (the same time in each experiment), and was then placed at 4° C. for several hours, or overnight, upon which the end-points were determined by observation of the deposit patterns. The first $\pm$ reaction, according to Salk's nomenclature, was regarded as end-point. In the absence of $\pm$ reaction, the end-point was calculated as the geometric mean of the last + and first — reaction.

The titre value was always calculated as the geometric mean of the end-points in the two simultaneous serial dilutions, and it has been recorded throughout as the 10-logarithm of the reciprocal of the final virus concentration, e.g. 1/20=1·30.

The chicken red cells were stored as 10 per cent suspension at 4° C. during a week. A 0·25 per cent red cell suspension was prepared each day and kept during the day at 4° C., chiefly to diminish the risk of bacterial growth, which may disturb the haemagglutination (von Magnus, personal communication).

The inhibitory activity of a test material was determined by comparing two titrations. In the one, the test material was used as diluent in the serial dilutions of the virus; in the other, the control, saline was substituted for the test material. The difference between the two titre values, expressed in log units, is a relatively sensitive measure of the inhibitory activity of the tested fluid.

The titrations in one and the same experiment were performed as far as practicable on the same day, in order to obtain results as comparable as possible.

EXPERIMENTAL.

The precision of haemagglutination titrations.

In order to estimate the precision of the haemagglutination titrations a larger number were performed during the course of one day. The results of four different series are listed in Table I.

The same virus suspension was used throughout and the same normal allantoic fluid in the two last experiments. Those titrations, where the virus was diluted in saline, were performed the same day; the red cell suspension, however, was stored during the day at different temperatures. In the one experiment it was kept at 4° C., in the other at room temperature. As Table I reveals, the result was not significantly affected by this difference. For this reason the standard deviation and its dispersion were calculated on the combined series.

The dispersion of the standard deviation $\left(\frac{\sigma}{\sqrt{2n}}\right)$ of the 70 titrations in

TABLE I.—*Deviations and Standard Deviations of Haemagglutination Titrations, the Virus Diluted in Saline and Normal Allantoic Fluid respectively.*

Diluent.	Number of titrations.	Mean of titre values in log units.	Deviation from the mean log. Least.	Greatest.	Mean.	Standard deviation. σ.	Standard deviation of σ. $\frac{\sigma}{\sqrt{2n}}$
Saline	35	3·90	+0·03	+0·19	±0·08	0·099	0·104 . 0·0085
	35	3·90	+0·03	+0·19	±0·09	0·109	
Normal allantoic fluid	30	1·83	+0·05	+0·13	±0·08	0·085	0·080 . 0·0075
	27	1·79	−0·06	+0·09	±0·07	0·075	

saline was as low as 0·0085, and for the titrations in normal allantoic fluid 0·0075. For this reason it is justifiable to consider as significant a difference of $\geqq 3 \cdot \sqrt{2} \cdot \sigma$ between two titres performed on the same day, and with the same red cell suspension. Approximating the standard deviation at 0·10, one may regard a difference of 0·42 log units as significant.

As a matter of fact among the author's tests only very occasionally did a difference occur between two duplicate titres of $>0 \cdot 30$ log units. As a rule, in these cases the dilution had been performed in turbid suspensions which rendered difficult the reading of the deposit patterns.

The relation between concentration of inhibitor and inhibition.

The specimens were diluted with saline solution in a twofold series. Each dilution was used as a diluent for the test virus in a haemagglutination titration. In Fig. 1 the titre values are plotted against dilution, both in terms of log values.

Line A is such a dilution curve obtained with a normal allantoic fluid, while B illustrates a preparation of the same, concentrated and partly purified by ultrafiltration. Each point on line A represents the mean of two haemagglutination titres, on B, the mean of three. The titration of the starting material (A) was performed two days after that of the concentrate (B).

As revealed in Fig. 1, the dilution curves present no straight lines. For evaluation of the concentration of inhibitor in a material treated in one way or another, a standard curve must therefore be recorded and the values calculated by interpolation. When, as in the present case, the dilution curves of two preparations do not run parallel, the specimens are obviously not directly comparable. Any attempt to compare the concentrations of inhibitor in two such preparations must necessarily introduce an approximation.

The dilution curve for the untreated allantoic fluid has at the beginning a slope of more than 45°, in agreement with the observation that a mixture of virus and normal allantoic fluid which does not haemagglutinate may become agglutinating on dilution with saline. This phenomenon has been dealt with earlier in connection with mumps virus (Beveridge, 1946) and Newcastle disease virus (Cunha *et al.*, 1947), and it was easily confirmed in reference to influenza virus.

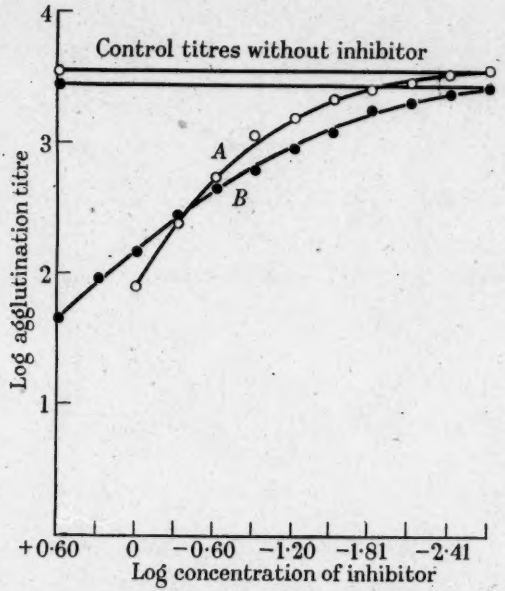

FIG. 1.—The inhibitor concentration of normal allantoic fluid taken as a unit. Relation between concentration of inhibitor and inhibition. Inhibitory material: A (o) a normal allantoic fluid, B (●) a preparation of the same fluid, partially purified and concentrated by ultrafiltration.

The inhibitory activity of normal allantoic fluid from embryos of various age.

Normal allantoic fluid from embryos varying in age was harvested in the usual manner. Each portion consisted of at least 20 eggs in order to avoid individual variations.

Fig. 2 shows the inhibitory activity, plotted against the age of the embryo.

The inhibitory activity was thus moderate at seven days, and increased considerably on further incubation. Fluids from embryos of eleven days and more had an inhibitory activity of about 2 log units.

The stability of the inhibitor at different temperatures.

The durability of the inhibitory activity of a normal allantoic fluid in storage without preservative is illustrated in Table II.

Each titre value was determined as the mean of 5 individual titrations.

TABLE II.—*Inhibitory Activity of a Normal Allantoic Fluid after standing for Different Periods of Time at 35° C., at Room Temperature and at 4° C.*

Storage conditions.	Log inhibitory activity.					
	2 days.	9 days.	16 days.	23 days.	1 month.	2 months.
35° C.	2·03	..	..	..	..	..
Room temperature	2·12	1·58	1·71	1·36	..	..
4° C.	2·20	1·79	1·86	1·49	1·58	1·21

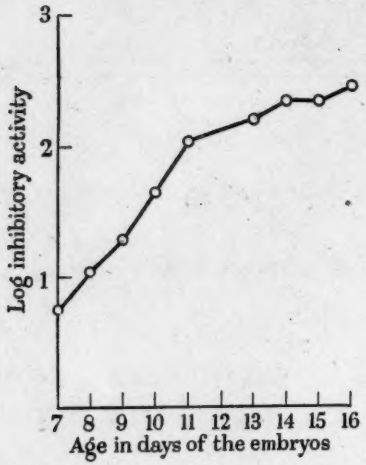

FIG. 2.—The inhibitory activity of the normal allantoic fluid from embryos of various ages.

Storage at two days gave no significant decrease in the inhibitory activity, but standing at room temperature for three weeks as well as at 4° C. during a period of two months brought about a diminution of approximately 1 log unit.

After freezing to −65° C. and subsequent thawing, a normal allantoic fluid lost its inhibitory potency and became instead slightly haemagglutinating. It was of no significance whether the thawing proceeded rapidly or slowly. There was always a fairly abundant precipitate.

The inhibitor manifested a fair thermo-resistance. Heating of a normal allantoic fluid to 65° C. for half an hour resulted in no significant decrease of the inhibitory activity. Boiling with subsequent immediate cooling in cold water gave a decrease of about 0·3 log units. Boiling in a water-bath for one hour diminished the inhibitory activity by 1 log unit.

The inhibitory activity of virus-infected allantoic fluid.

Due to the heat-resistance of the inhibitor it was found possible to examine virus-infected haemagglutinating suspensions for the presence of inhibitor. The virus haemagglutinins are completely inactivated by the boiling process, while the inhibitor is only affected relatively little.

In the experiment illustrated in Fig. 3, a batch of eggs 11 days old was inoculated with an infective allantoic fluid diluted 10^{-6}. After 12 hours' incubation at 35° C. the allantoic fluid was harvested from 20 surviving eggs, cooled to 4° C. during a few hours to avoid bleeding. In the same manner, sets of 20 eggs were harvested after 24, 36 and 48 hours respectively. A determination of the haemagglutination titre and the inhibitory activity following the heat-inactivation of the haemagglutinins (boiling and cooling) was made on each sample, all titres in duplicate. In Fig. 3 the mean titre values are reproduced.

As seen, the inhibitory activity gradually diminished during the incubation after inoculation. When the haemagglutination titre had reached its maximum,

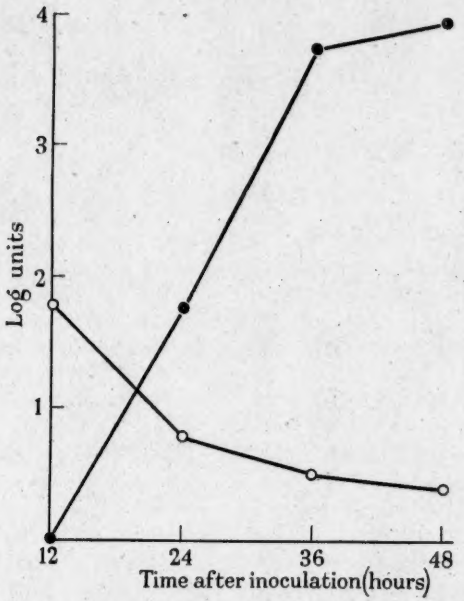

FIG. 3.—Haemagglutination titres and inhibitory activity of the allantoic fluid during incubation after inoculation. The inhibitory activity was determined after heat inactivation of the haemagglutinins.

●——● = haemagglutination titres.
o——o = inhibitory activity.

the inhibitory activity was scarcely significant. A slight residue of the activity has, however, regularly occurred in infected fluids.

The decrease in the allantoic fluid's inhibitory activity may presumably be a result of the presence of virus in the allantoic fluid. This assumption is not improbable when, as a matter of fact, the inhibitory activity can be destroyed *in vitro* by addition of virus infected allantoic fluid. Such an experiment is summarized in Table III.

TABLE III.—*Demonstration* in vitro *of Inhibitor Destroying Capacity of Virus Infected Fluid.*

Diluent.*	Log agglutination titre.
Saline	3·56
PR8 allantoic fluid	3·18
Normal allantoic fluid diluted 1/2 in saline	2·43
PR8 allantoic fluid + normal allantoic fluid, equal parts	3·11
PR8 allantoic fluid, boiled before mixing + normal allantoic fluid, equal parts	2·43

* All diluents except saline were heat inactivated (boiled and immediately cooled) at once after mixing.

When measured amounts of fluid containing active virus were mixed with normal allantoic fluid, little inhibitory activity could be demonstrated in the mixture after heat inactivation, immediately after mixing, of the haemagglutinins. If, on the other hand, the virus-containing fluid was heat-inactivated before mixing, the mixture was found to possess an inhibiting activity corresponding to the amount of normal fluid added. This subject will be treated further in a forthcoming paper.

The particle size of the inhibitor.

Gradocol filtration tests.—In order to get some idea as to the size of inhibitor particles, some preliminary Gradocol filtrations of a normal allantoic fluid were performed.

The fluid was filtered through a membrane with an average pore diameter of 500 mμ., subsequently 125 mμ. and finally 32 mμ. All the material which passes through a filter was collected. Each fraction was titrated on inhibitory activity. The result of this test is seen in Table IV.

TABLE IV.—*Gradocol Filtration Experiments.*

Diluent.	Log agglutination titre.
Saline	3·41
Normal allantoic fluid	1·05
Same filtered through 500 mμ.	1·65
,, ,, ,, 125 mμ.	3·18
,, ,, ,, 32 mμ.	3·26

It was found that a great deal of the inhibitory activity passed through a 500 mμ. membrane, while the fluid after being filtered through the 125 mμ. membrane contained no significant inhibitory activity. This indicates that the said activity is connected with relatively large particles of an order of magnitude similar to that of the virus particles.

It was also possible to employ ultrafiltration (Seibert, 1928) for concentration and partial purification of the inhibitor.

Differential centrifugation.—In order to confirm the results of the Gradocol filtration experiments a normal allantoic fluid was fractionated by centrifugation.

The fluid was firstly spun at 27,000 r.p.m. for one hour. The supernatant was removed in two portions (SI = the upper 4/5, SII = the lower 1/5). The deposit was resuspended in saline to the original volume (DI) and half of the suspension was centrifuged up-and-down to 10,000 r.p.m. The supernatant was again removed in portions (upper 3/4 = SII, lower 1/4 = SIV). The deposit was resuspended in saline to the original volume (DII). Activity titrations of the usual kind were performed on each fraction. In Table V the agglutination titres are listed. The third column shows the amount of inhibitor demonstrable in the respective fractions, calculated approximately from a dilution curve as given in Fig. 1.

The main part of the inhibitor was found in the deposit after centrifugation for 1 hour at 27,000 r.p.m. Only about 10 per cent appeared in the coarse matter fraction.

TABLE V.—*Fractionation of a Normal Allantoic Fluid by Differential Centrifugation.*

Diluent.	Log agglutination titre.	Approximate recovery of inhibitor in different fractions.
Saline	3·48	..
Normal allantoic fluid	1·20	100 per cent
SI Supernatant after 27,000 r.p.m.	2·58	
SII 60 min.	2·43	10 ,,
DI deposit after 27,000 r.p.m. 60 min.	1·73	50 ,,
SIII deposit cleared from coarse	1·98	..
SIV matter	1·58	..
DII coarse matter	2·63	10 per cent

Determination of the sedimentation constant of the inhibitor.—The sedimentation constant of the inhibitor particles was determined by means of centrifugation in the separation cell (Tiselius, Pedersen and Svedberg, 1937) of a partially purified concentrate, the calculation being based upon the rate of decrease in inhibitory activity of the contents in the inner compartment of the cell, and compared with the results of an ordinary analytical centrifugation with optical registration of moving boundaries.

A normal allantoic fluid was washed with buffered saline (0·85 per cent saline 9 parts + 0·1 M phosphate buffer pH 7·0, 1 part) and was concentrated 50 times by ultrafiltration (Seibert, 1928). The highly viscous preparation (relative viscosity 1·87) was centrifuged at 3500 r.p.m. for 40 minutes, and the supernatant which, in a preliminary titration, was found to contain practically the whole of the original inhibitory activity, was used in the following experiments.

Some of the material was centrifuged in the separation cell at 14,000 r.p.m. for 105 minutes. The contents of the inner and the outer compartment were collected separately. The material was diluted 1/50 (= the concentration of the original normal allantoic fluid), and was employed in the usual manner as diluent in the agglutination titration of a test virus, simultaneously with a series of titrations of different concentrations of uncentrifuged material. All the titrations were performed in triplicate. The result of the titrations of the uncentrifuged material is graphically illustrated as curve B in Fig. 1, where the lowest dilution is 12·5 times the dilution of the concentrate (= 4 times the concentration in relation to the original normal allantoic fluid.)

The titre of the content of the inner compartment revealed an increase from 2·17 to 2·88. Through interpolation in Fig. 1, this is seen to correspond to a residue of 9 per cent of the inhibitor in the inner cell. Judging from this a sedimentation constant of the inhibitor of 125 S could be calculated.

Analogously the quantity of inhibitor in the resuspended content of the outer compartment had increased to double. On calculating the total inhibitor content of the cell, a definite deficit was found in comparison with the control. This is often observed on centrifugation of high molecular gel-forming substances. Part of the material is adsorbed in the filter-paper which covers the perforated membrane between the outer and inner compartments, and some of the sediment

at the bottom of the cell will not be resuspended. Estimations of the concentration in the outer compartment will, therefore, as a rule yield too low values, which renders this figure unsuitable as a basis for calculations of sedimentation velocity (Gard, 1943).

When performing an ordinary analytical centrifugation at 14,000 r.p.m., of the same material, with optical registration of the moving boundaries, two components were observed, one slowly sedimenting and homogeneous, S_{20} = 11 S, the other faster and moderately homogeneous, S_{20} = 113 S.

With regard to experimental error the values 113 and 125 are in satisfactory agreement. The inhibitory effect in this material should thus be connected with particles having a sedimentation constant of about 120 S.

The same preparation, but only 20 times concentrated (= 2/5 of the former), was centrifuged in a corresponding manner 74 minutes at 14,000 r.p.m. in the separation cell. From the result a sedimentation constant of S_{20} = 170 S could be calculated.

Gard and von Magnus (1947) and Gard, von Magnus and Svedmyr (1947) have purified both components mentioned in normal allantoic fluid by means of differential centrifugation. A description of such a preparation will be given in the following, as the purified material was used by the author in some experiments about to be reported.

Nineteen litres of normal allantoic fluid were washed and concentrated by ultrafiltration to 1440 ml. to facilitate the handling of the same during the centrifugation. The preparation was centrifuged 90 minutes at 27,000 r.p.m., after resuspension of the deposit coarse matter was removed by centrifuging up-and-down to 10,000 r.p.m., upon which the same cycle was repeated. The final concentrate was taken up in 0·1 M phosphate buffer pH 7·0 to 1/1000 of the original volume. The preparation was highly viscous (relative viscosity 3·21).

The analytical centrifugation of this preparation in 1/2 dilution disclosed a component with S_{20} = 83 S. No other component was detectable. Dilution 1/5 gave the value of S_{20} of 180 S. If these sedimentation constants are "corrected" for viscosity by multiplication by the relative viscosity of the respective solutions (1·93 and 1·34 respectively) the values 160 and 241 are obtained. Consequently the dependence of sedimentation velocity on concentration cannot be explained as being solely due to the viscosity of small invisible quantities of other components (e.g. " 11 S " component) in the preparation.

Seeing that even in the author's above reported tests possibility is lacking to adequately determine the effect of other components in the preparation, on the sedimentation constant, it is futile to attempt to " correct " the constant by multiplying with the relative viscosity of the preparation. With the guidance of the results of Gard and von Magnus the sedimentation constant at the concentration 0 may be estimated at about 200 S.

The inhibitory activity of highly purified preparations of the " 200 S " component.

Fig. 4 illustrates two " dilution curves " which were produced in the same way as the curves in Fig. 1. Line B represents the above described preparation of the normal component " 200 S " purified by centrifugation, line A the normal allantoic fluid which was the starting material of the said preparation. The titration of the starting material was performed two days after that of the concentrate. Each titre was in duplicate.

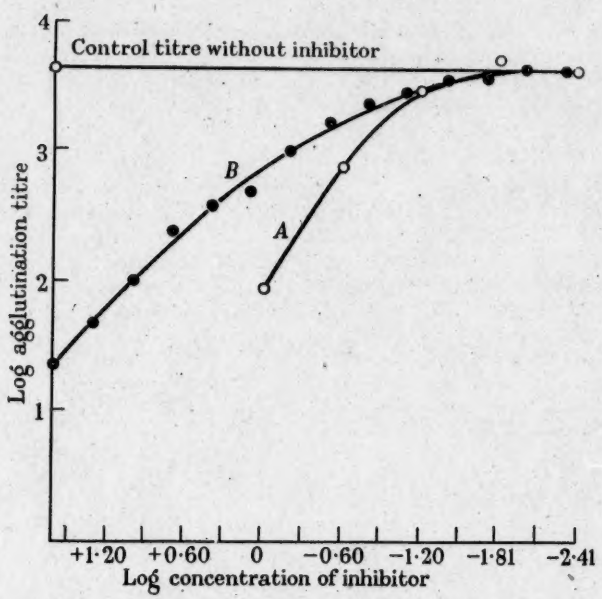

FIG. 4.—The inhibitor concentration of normal allantoic fluid taken as a unit. Relation between concentration of inhibitor and inhibition. Inhibitory material: A (o) a normal allantoic fluid, B (●) a highly purified preparation of the " 200 S " component, obtained from the same fluid.

As in Fig. 1, the curves do not run parallel. The dilution curve for the untreated material shows a steeper slope at the beginning. For this reason any exact estimation of the yield of inhibitor is impossible. By way of a suggestion the phenomenon might be due either to the effect of several factors in the crude allantoic fluid or, possible, to some change in the inhibitor during the purification process.

On the contrary, the curves for the preparation purified by ultrafiltration (B, in Fig. 1) and for the purer, differentially centrifuged preparation (B, in Fig. 4) run almost parallel during the greatest part of their courses. As the concentrations met with in the experiment with the separation cell described above fall within the range where the two curves run almost parallel, the various fractions in that experiment may be considered directly comparable. It is therefore justifiable to base—as done—the calculations of the concentrations of the inhibitor on interpolation in the dilution curve for the uncentrifuged control material (B, in Fig. 1).

Observations on virus-inhibitor mixtures.

Von Magnus (personal communication) found that an immediate flocculation occurred when he mixed solutions of virus and the normal component " 200 S " both purified and concentrated by means of differential centrifugation. In balanced mixtures neither the supernatant nor the deposit haemagglutinated. A similar experiment made by the author will be illustrated in the following:

2·5 ml. of a 1/10 dilution of the same normal component preparation as was employed in the previous experiment (accordingly 100 times concentrated) was mixed with the same volume of PR8 virus suspension, purified and concentrated 50 times by differential centrifugation by Gard and von Magnus. Each preparation was suspended in 0·1 M phosphate buffer pH 7·0. The mixture was allowed to stand for 15 minutes at + 25° C., and then centrifuged in an angle-centrifuge at 4400 r.p.m. for 15 minutes. A considerable sediment was deposited from the mixture of the previously stable preparations. The sediment was washed in 5 ml. of saline to remove the phosphates, and after further centrifugation at 3800 r.p.m. for 15 minutes the deposit was resuspended in 2 ml. of buffered saline containing 0·01 M $CaCl_2$. This preparation was employed in an experiment which will be reported in a subsequent paper, and only the titration results of the various fractions will be given here. See Table VI.

TABLE VI.—*Precipitation by Mixing Virus and Inhibitor.*

	Log agglutination titre.
Purified virus concentrate	4·24
Virus + inhibitor :	
Supernatant after 4400 r.p.m. 15 min. . .	1·28
Deposit washed with saline :	
Supernatant after 3800 r.p.m. 15 min. .	0·45
Deposit „ „ „ . .	0·83

The test, which confirms the observation of von Magnus, reveals that a mixture of normal component and virus particles can produce a precipitate. The major part of the haemagglutinins were neutralized by the inhibitor.

Effect of varying the order of mixing virus, inhibitor and red cells.

A. The virus dilution was prepared in normal allantoic fluid from embryos 11 days old, and the red cell suspension was added 15 minutes later.

B. The virus dilution was prepared in the red cell suspension and the normal allantoic fluid was added 15 minutes later.

C. The red cell suspension and the normal allantoic fluid were mixed in equal proportions and allowed to stand for 15 minutes, after which the virus dilution was prepared in the mixture.

Saline was substituted in the controls for normal allantoic fluid. The results are illustrated in Table VII.

TABLE VII.—*Effect of Varying the Order of Mixing Virus, Inhibitor and Red Cells.*

	Log agglutination titre.	
	Test.	Control.
A. Virus + inhibitor + red cells . .	1·45	3·26
B. Virus + red cells + inhibitor . .	2·65	3·33
C. Red cells + inhibitor + virus . .	1·68	3·33

The inhibition was considerably weaker when the inhibitor was added last.

DISCUSSION.

The filtration and centrifugation experiments indicate that at least the main part of the inhibitory activity of normal allantoic fluid is connected with particles with a sedimentation constant of about 200 S.

A similar component in normal allantoic fluid has been earlier demonstrated by Knight (1944), Gard and von Magnus (1947), and Gard, von Magnus and Svedmyr (1947). Knight called attention to particles with an estimated sedimentation constant of approximately 170 S. He also found that the yield of this substance increased with the age of the embryo. Gard and von Magnus discovered a corresponding component in normal allantoic fluid as well as in infected liquid harvested after 6 hours' incubation. On the other hand, they were not able to demonstrate this component in fluids which contained appreciable amounts of virus. The occurrence of such a component would seem, in other words, to run parallel with the behaviour of the inhibitory activity. Some of the highly purified preparations of this normal component obtained by Gard and von Magnus, when titrated, were found also to possess a considerable part of the fluid's original inhibitory activity.

Strong evidence for the assumption that the inhibitor is identical with this normal component is finally to be found in the precipitation reaction which occurs when purified preparations of virus and of the normal component are mixed. The phenomenon was more closely analysed by Gard, von Magnus and Svedmyr (1947) with physico-chemical methods (analytical centrifugation). The components apparently reacted with each other in definite proportions. Accordingly, if one component was present in excess, only this one could be demonstrated in the solution, after the precipitate was removed, and then in a concentration which, in comparison with the original, was reduced in proportion to the amount of the other component added.

These tests indicate that the inhibitor acts against the haemagglutinin rather than against the receptor. This assumption is further supported by the observation that the inhibitory activity appreciably diminished if virus and the red cells were mixed prior to the addition of the inhibitor.

When influenza virus was cultivated in the allantoic sac, the inhibitory activity diminished in the allantoic fluid gradually and accordingly as the virus increased. As a physico-chemical manifestation thereof it was impossible to demonstrate any " 200 S " component when the virus had multiplied. Infected allantoic fluid had even *in vitro* an immediate inactivating or blocking effect on the inhibitory activity when added to normal allantoic fluid. Both these phenomena might assumably be considered as the result of the effect of virus on the inhibitor.

The problems concerning virus-inhibitor interaction will be more directly treated in a forthcoming paper.

SUMMARY.

Normal allantoic fluid has an inhibiting effect on influenza virus haemagglutination. The inhibitory activity is moderate in the allantoic fluid from embryos 7 days old, and increases considerably on further incubation.

After the inoculation of influenza virus into the allantoic cavity, the inhibitory activity gradually decreases, and when the haemagglutination titre has reached its maximum little inhibitory activity can be observed.

The inhibitor is presumably identical with a particulate component which is present in normal allantoic fluid, and which has a sedimentation constant of approximately 200 S.

The results indicate that the inhibitor acts against the haemagglutinin rather than against the receptor.

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STUDIES ON A FACTOR IN NORMAL ALLANTOIC FLUID INHIBITING INFLUENZA VIRUS HAEMAGGLUTINATION. VIRUS-INHIBITOR INTERACTION.

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HIRST (1942) showed that influenza virus on incubation with red cells destroys the receptors. Under similar conditions the inhibitory activity of red cell extracts (Friedewald, Miller and Whatley, 1947; Bovarnick and de Burgh, 1947; de Burgh, Pen-Chung, Howe and Bovarnick, 1948), and of normal serum (Burnet, McCrea and Anderson, 1947; Burnet, 1948; Hirst 1948) is likewise destroyed.

The present author, in previous papers (Svedmyr, 1947*a*, 1947*b*, 1948), has described the inhibitor present in normal allantoic fluid obtained from chick embryos. The observations indicated that at least the main part of the inhibitive capacity of normal allantoic fluid on influenza virus haemagglutination is connected with particles having a sedimentation constant of about 200 S, and that the inhibitor acts against the haemagglutinin rather than against the receptor. Furthermore, the inhibitory activity of the allantoic fluid was found to decrease gradually to an inconsiderable minimum during multiplication in the embryo of influenza virus. *In vitro* tests revealed that virus-infected allantoic fluid when added to equal volumes of normal allantoic fluid immediately interferes with the inhibitory activity.

In this paper further observations will be put forward with reference to virus-inhibitor interaction.

MATERIALS AND METHODS.

Virus preparations.—The PR8 strain of influenza virus was used throughout. In addition, the Lee strain of influenza virus and mumps virus were included in one experiment. The strains were propagated in the allantoic cavity of chick embryos, as previously described (Svedmyr, 1948). The mumps strain was kindly supplied by Dr. G. Henle, and maintained in this laboratory by allantoic passages by Dr. H. Lundbäck.

As a rule, virus preparations containing formalin and merthiolate, both 1/10,000, and stored at 4° C. were used for determination of inhibitory activity. As will be shown later, admixture of preservatives in these concentrations was of no significance to virus-inhibitor interaction.

Normal allantoic fluid.—As in the previous work, allantoic fluid from chick embryos 13 days old was employed. The fluids were stored at 4° C., as a rule with the addition of merthiolate 1/10,000 as preservative. In that case, however, titrations had to be read the day they were performed, as prolonged exposure to merthiolate caused haemolysis.

Haemagglutination tests.—The methods were described and their precision analysed in a previous paper (Svedmyr, 1948).

Chick embryo titrations.—Virus was diluted in tenfold steps in 0·1 M phosphate buffer pH 7·0, and each dilution in the amount of 0·1 ml. per egg was inoculated into 3–10 eggs. In some experiments where the risk of bacterial contamination was obvious, 500 units of penicillin and 1 mg. of streptomycin per ml. were added to the buffer (Florman, Weiss and Council, 1946 ; McKee and Hale, 1947). Subsequent to incubation at 35° C. for 48 hours the eggs were chilled overnight at 4° C. The allantoic fluids were then tested individually for haemagglutinins. The 50 per cent end-point was calculated according to Weiss (1948) by means of his abridged table of probits for use in the graphic solution of the dosage-effect curve.

EXPERIMENTAL.

The capacity of influenza and mumps viruses to affect the inhibitory activity of normal allantoic fluid.

In the present experiments the inhibitor-destroying capacity (IDC) of a virus suspension was determined as follows:

Normal allantoic fluid was incubated at 35° C. with small amounts of fluid infected with the PR8 and Lee strains of influenza virus and the strain of mumps virus mentioned above. The proportions of the reagents in the different mixtures were as stated in Table I. Samples were removed after 0, 4 and 20 hours' incubation, heated in a water-bath to 65° C. for 30 minutes in order to interrupt the process, and stored at 4° C. until titrations of the residual inhibitory activity of all samples could be performed simultaneously. The results summarized in Table I show that a gradual destruction of the inhibitor took place.

TABLE I.—*Decrease in the Inhibitory Activity of a Normal Allantoic Fluid by Incubation with small amounts of Allantoic Fluid infected with Virus. Samples were removed after different Periods of Time, and Tested for Residual Inhibitory Activity after Heating to 65° C. for 30 minutes.*

			PR8.	Lee.	Mumps.
	Strains of virus.				
	Concentration of virus fluid		1/300.	1/150.	1/150.
Residual inhibitory activity*	Hours of incubation	0	1·96	1·81	1·81
		4	1·81	1·51	1·66
		20	1·36	1·21	1·21

* Titre value of the saline control of the test virus 4·09.

In order to ascertain whether the virus particles themselves or some other constituent of the infected allantoic fluid were responsible for the inactivation of the inhibitor, an experiment with a virus preparation purified by differential centrifugation was carried out as follows:

An infective PR8 allantoic fluid, after clarification by centrifugation at 3500 r.p.m. for 10 minutes, was centrifuged at 12,500 r.p.m. for 2 hours. The sediment was resuspended in 0·1 M phosphate buffer pH 7·0. This suspension was clarified by centrifugation up-and-down to 10,000 r.p.m., and then again subjected to centrifugation at 12,500 r.p.m. for 2 hours. The final deposit was resuspended

in phosphate buffer. The centrifugations were performed in the cold room during the course of one day by means of a Servall superspeed angle centrifuge.

In spite of these precautions most of the infectivity was destroyed, as shown in Table II. Whereas the haemagglutination titre indicated a virus concentration in the purified product of about 8 times that of the original fluid, the infectivity had decreased to less than 1 per cent. On the basis of the relative virus concentrations, calculated from the haemagglutination titres, the IDC of the purified preparation can be estimated at about 20 per cent of the original (Table IV). On account of the limited accuracy of the methods of assay the last-mentioned figure represents only a rough approximation. In any event the preparation had retained proportionately much more of the IDC than of the infectivity originally present.

As will be shown later, heat inactivation of infectivity also causes a certain loss of IDC. Provided the virus inactivation was caused by the high temperature produced in the centrifuge, it must be inferred that the IDC as well might have been affected to a considerable extent. As a matter of fact a comparison with Table IV reveals that the decrease in IDC observed corresponds tolerably well with the actual loss of infectivity. The IDC of the supernates from the centrifugations were not determined, so the amount of inhibitor-destroying agent removed with these fractions cannot be estimated. The data presented are, however, not inconsistent with the assumption that the IDC is connected with the virus particles.

Table II also shows that if the destruction of the inhibitor is driven sufficiently far, haemagglutinins are released.

The effect of formalin and heat treatment on the biological activities of the virus.

Preliminary tests showed that an allantoic fluid infected with PR8 virus and stored for 9 months at 4° C with formalin and merthiolate, both 1/10,000, and which was not infective for eggs in serial passage, had retained the haemagglutination capacity as well as the power to inactivate the inhibitor. This phenomenon was further studied with virus inactivated in various ways.

A. *Formalin treatment of virus.*—To different portions of an infective allantoic fluid formalin was added in concentrations 1/10, 1/100, 1/1000 and 1/10,000 respectively and merthiolate 1/10,000. The fluids were stored for 6 days at 4° C. The excess formalin was then removed by ultrafiltration (Seibert, 1928) and washing on the filter. The 50 per cent chick embryo infectivity end-point, the haemagglutination titre and the effect of normal allantoic fluid on the test material expressed as inhibitory activity was determined for the various preparations. Moreover, the IDC of the respective virus suspensions was determined. The results are given in Table III.

The experiment shows that cautious treatment with formalin can destroy completely the infectivity, while the IDC is only slightly reduced and the haemagglutination capacity not affected to any appreciable extent.

B. *Heat treatment of virus.*—Portions of an infective allantoic fluid were heated in a water-bath at 56° C. and 50° C. respectively for 30 minutes. The same tests as in the previous experiment (A) were performed on these preparations as well as on the untreated material. The results are listed in Table IV.

It is obvious that the infectivity was destroyed more rapidly than the IDC, which in its turn proved more labile than the haemagglutination power. Heat-

TABLE II.—*Properties of PR8 Virus Purified by Centrifugation Compared with those of the Starting Material.*

Virus material		PR8 allantoic fluid.	Purified concentrate.	
50 per cent chick embryo infectivity end-point (3 eggs per group)		5·5	2·8	
Haemagglutination titre		3·78	4·69	
Effect on the virus of normal allantoic fluid, expressed as inhibitory activity		2·25	2·41	
Residual inhibitory activity of normal allantoic fluid incubated with the virus.*	Concentration of virus	1/100.	1/500.	Control without virus.
	Hours of incubation 0	1·95	1·95	1·80
	Hours of incubation 15	+	0·45	..
	Hours of incubation 20	+	0·60	1·95

\+ = positive haemagglutination of the tested fluid. Titre in saline 0·83 and 1·13 respectively.

* Normal allantoic fluid was incubated at 35° C. with different amounts of the virus. Samples removed after different periods of time and tested for residual inhibitory activity after heating to 65° C. for 30 minutes. Titre value of the saline control of the test virus 3·48.

TABLE III.—*Properties of Virus-Infected Fluids Treated with Formalin.*

Infected fluid treated with formalin in the following concentrations	1/10.	1/100.	1/1000.	1/10,000.
50 per cent chick embryo infectivity end-point (3 eggs per group)	Negative	Negative**	Negative	0·5***
Haemagglutination titre	0·68	2·88	3·63	3·78
Effect on the virus of normal allantoic fluid, expressed as inhibitory activity	—	1·90	2·35	2·20

Residual inhibitory activity of normal allantoic fluid incubated with the formalinized virus.*	Concentration of virus	1/10.	1/100.	1/10.	1/100.	1/10.	1/100.	1/10.	1/100.	Control without virus.
	Hours of incubation 0	1·88	1·88	1·88	1·88	1·58	1·88	1·28	1·73	1·73
	Hours of incubation 4·5	1·88	1·88	1·88	1·88	+	1·58	+	0·83	..
	Hours of incubation 20	1·88	1·88	1·58	1·88	+	0·98	+	+	1·88

Formalin in different concentrations was added to allantoic fluid infected with PR8 virus. After standing at 4° C. for 6 days the fluids were dialysed and tested for the different properties listed above.

** The allantoic fluid from one egg inoculated with undiluted fluid haemagglutinated. On dilution 1/2, however, it was negative and serial passages in the allantoic cavity were negative. Thus it proved that the haemagglutinaton was from the inoculated haemagglutinins.

*** The fluids from three eggs inoculated with undiluted fluid were positive and proved positive on serial passage.

* See note, Table II. Titre value of the saline control of the test virus 3·71.

\+ = positive agglutination of the tested fluid.

treated virus which haemagglutinated but had lost its power to inactivate the inhibitor was still able to combine with the same, and was more than active virus inhibited by normal allantoic fluid.

Inactivation test with von Magnus' non-infective virus modification.

Von Magnus (1947*a*, 1947*b*) has demonstrated that in serial passages in the allantoic sac of undiluted PR8 infected allantoic fluid, harvested at the time for maximum virus activity (i.e. 20–24 hours), the infectivity titre greatly decreased from passage to passage, and generally attained its minimum of 1/10,000 or less of the original titre after 3 generations. The immunization and haemagglutination capacities, however, reached about the same levels as under standard conditions. The occurrence of auto-interference in mice indicated that two kinds of reciprocally competitive viruses were formed at the passages.

Gard and von Magnus (1947) and Gard, von Magnus and Svedmyr (1947) analysed the phenomenon by physico-chemical methods, and were able to show that a component with a sedimentation constant of about 500 S appeared in increasing amounts from the first to the third passage, while the "standard virus" with a sedimentation constant of about 700 S simultaneously decreased. The sum total of the two components, however, remained relatively constant. Gard and von Magnus are of the opinion that the 500 S component is an incomplete virus particle, which has the capacity to produce specific immunity and the power to haemagglutinate but lacks infectivity.

The results presented in this paper suggested the investigation of the inhibitor-destroying capacity of this virus modification.

The starting material in the experiment was an ordinary standard passage, i.e. allantoic fluid from eggs inoculated with infective fluid diluted 10^{-6}. This was passed undiluted through 5 generations in the allantoic sac. The eggs, 5 in each group, were incubated for 22–24 hours at 37° C., the fifth passage, however, for 36 hours. The same tests as in the previous experiments were performed on the standard passage, the third, fourth and fifth passages. Haemagglutination titres were obtained also for the first and second passages (Table V).

In relation to the haemagglutination titre, which, in this case, must be regarded as representing a measure of the total virus concentration, no reliable difference existed between the IDC of the standard passage and the various serial passages. Thus it is obvious that the virus, even in the least infective passages, retained at all events the main part of its capacity to destroy the inhibitor.

Quantitative aspects of virus-inhibitor interaction.

It was previously shown (Svedmyr, 1947*b*, 1948) that when measured amounts of infective and normal allantoic fluids were mixed, very little inhibitory activity could be demonstrated in the mixture after immediate heat inactivation of the haemagglutinins. If, on the other hand, the virus-containing fluid was heat-inactivated prior to the mixing, the mixture was found to possess an inhibitory activity corresponding to the amount of normal fluid added.

In order to study this phenomenon further, the haemagglutination titre and the inhibitory activity in mixtures of virus and inhibitor in various proportions were determined.

TABLE IV.—*Properties of Virus-Infected Fluids Treated by Heating.*

Infected allantoic fluid heated to the following temperatures for 30 minutes	56° C.	50° C.	Not heated.
50 per cent chick embryo infectivity end-point (10 eggs per group)	1·9	3·5	6·6
Haemagglutination titre	3·78	3·93	3·93
Effect on the virus of normal allantoic fluid, expressed as inhibitory activity	2·95	2·20	2·25

Residual inhibitory activity of normal allantoic fluid incubated with the heated virus.*

Concentration of virus		1/10.	1/100.	1/10.	1/100.	1/100.	1/400,	1/1600.	1/6400	1/25,600.	Control without virus.
Hours of incubation	0	2·25	2·25	1·50	2·25	2·10	2·25	2·25	2·32	2·25	2·25
	3	2·25	2·25	+	1·80	1·50	2·10	2·25	2·25	2·25	..
	23·5	1·95	2·25	+	+	+	0·90	1·80	2·10	2·25	2·25

* See note, Table II. Titre value of the saline control of the test virus 3·93.
+ = positive agglutination of the tested fluid.

TABLE V.—*Properties of Serial Passages of Undiluted Virus Fluids, Harvested at the time of Maximum Virus Activity.*

Virus material	"Standard passage."	Passage:— 1	2	3	4	5
50 per cent chick embryo infectivity end-point (6 eggs per group)	7·6	..	..	2·9	4·0	6·4
Haemagglutination titre	4·32	4·09	4·17	4·09	3·03	3·78
Effect on the virus of normal allantoic fluid, expressed as inhibitory activity	2·59	2·43	2·44	2·36	1·97	2·20

Residual inhibitory activity of normal allantoic fluid incubated with the tested virus passage.*

Concentration of virus		1/100.	1/400.	1/1,600.	1/6,400.			1/10.	1/100.	1/10.	1/100.	1/10.	1/100.	Control without virus.
Hours of incubation	0	1·50	1·65	1·65	1·65	..	..	0·45	1·35	1·35	1·50	0·75	1·50	1·50
	3·5	1·20	1·50	1·65	1·65	..	..	+	0·60	0·60	1·35	+	0·90	..
	20	·0·08	1·05	1·35	1·35	..	..	+	0·30	0·30	1·05	+	0·45	1·50

* See note, Table II. Titre value of the saline control of the test virus, 3·78.
+ = positive agglutination of the tested fluid.

A virus-infected allantoic fluid with intact inhibitor-destroying capacity was mixed with normal allantoic fluid in the proportions indicated on the abscissa in Fig. 1. Both the fluids when mixed had a temperature of about 4° C. The mixtures were immediately placed in a water-bath at 65° C. for 15 minutes, and were shaken for the first two minutes in order to make the temperature rise as speedily as possible. Preliminary tests had revealed that 15 minutes at 65° C. affected the haemagglutination titre but little while the IDC was eliminated.

Haemagglutination capacity (Fig. 1, line A) as well as inhibitory activity (line B) were determined. Moreover, the inhibitory activity, residual after destruction of the haemagglutinins by bringing the mixture to the boiling point, was determined (line C).

In parallel tests the virus suspension was heated for 15 minutes at 65° C. before being mixed with the normal allantoic fluid. The mixtures were then titrated, without further heating. Line F represents the haemagglutination titre, G the inhibitory activity without boiling, and H after boiling.

From the haemagglutination titre value of the virus-infected fluid, also heated for 15 minutes at 65° C., a control curve, D, was calculated. The in-

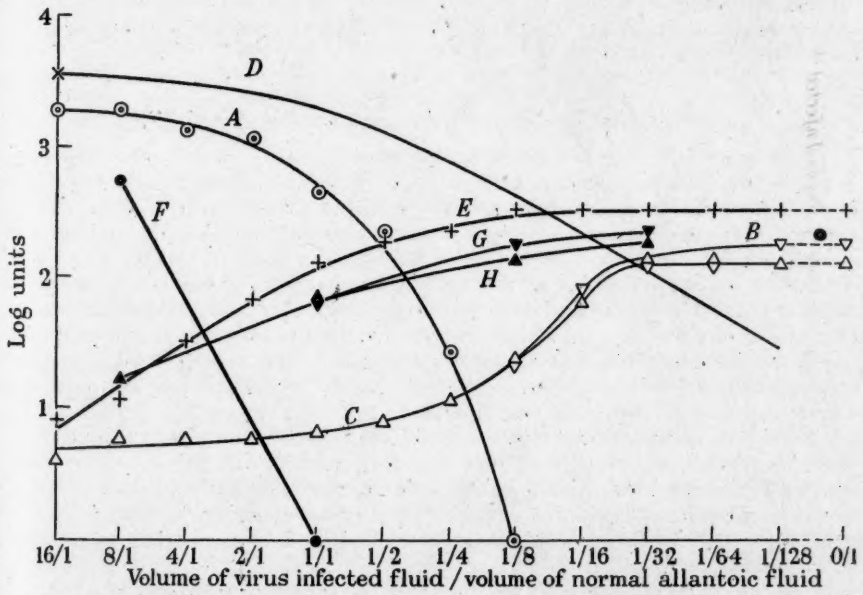

FIG. 1.—Haemagglutination titre and inhibitory activity of mixtures of virus-infected fluid and normal allantoic fluid in various proportions.

A (○): active virus + inhibitor, 65° C. 15 minutes. Haemagglutination titre.
B (▽): „ „ + „ „ „ „ Inhibitory activity.
C (△): „ „ + „ „ „ „ boiled. Inhibitory activity.
D (×): virus 65° C. 15 minutes + saline. Haemagglutination titre.
E (+): saline + inhibitor. Inhibitory activity.
F (●): virus 65° C. 15 minutes + inhibitor. Haemagglutination titre.
G (▼): „ „ „ „ + „ Inhibitory activity.
H (▲): „ „ „ „ + „ boiled. Inhibitory activity.

hibitory activity of the unheated normal allantoic fluid in concentrations as indicated on the abscissa was determined experimentally (line E).

The inhibitory activity (line C) of mixtures which were heated to 65° C. and then boiled concurs closely with the corresponding line B of the same materials heated to 65° C., but not boiled. Likewise line H (boiled mixtures) coincides with line G (inhibitor not heated). It is thus obvious that neither heating to 65° C. nor boiling of the inhibitor has had any appreciable effect on the course of the curves.

The addition of active virus to normal allantoic fluid was accompanied by a considerable reduction of the inhibitory activity even when the period of action was made as short as technically possible and, consequently, the previously mentioned destruction of the inhibitor, which proceeds at a fairly low rate, was negligible. On the other hand, when virus was heated prior to contact with the inhibitor, the inhibitory activity of the mixtures (G and H) was scarcely affected significantly.

A comparison between curves A and F shows that, even under the conditions of this experiment, i.e. heating of the reagents immediately after mixing, pre-heated virus is more sensitive to the action of the inhibitor than is active virus.

In previous papers (Svedmyr, 1947*a*, 1948) reactivation or dissociation on dilution of nonagglutinative mixtures of active virus and normal allantoic fluid was mentioned. It might be pointed out that a similar phenomenon was not observed in heated mixtures.

The effect on the inhibitor of a culture filtrate of Clostridium welchii.

The report of Burnet, McCrea and Stone (1946) that certain bacterial enzymes were able to destroy the receptors of red cells suggested the investigation of the effect of *Cl. welchii* filtrate on the inhibitor. The result of such an experiment was summarized in a previous paper (Svedmyr, 1947*b*). The details were as follows:

2·5 ml. of a preparation of the "200 S component" from normal allantoic fluid (Svedmyr, 1948), purified and concentrated 100 times by differential centrifugation, was mixed with the same volume of a PR8 virus suspension, purified and concentrated 50 times by differential centrifugation, both preparations suspended in 0·1 M phosphate buffer pH 7·0. Both preparations were kindly supplied by Gard and von Magnus.

The mixture was allowed to stand for 15 minutes at 25° C., and was then spun in an angle centrifuge at 4400 r.p.m. for 15 minutes. The heavy sediment was washed in 5 ml. of saline for elimination of the phosphates, and after further centrifugation for 15 minutes at 3800 r.p.m. the deposit was resuspended in 2 ml. of buffered saline containing 0·01 M $CaCl_2$. This suspension was divided into three parts, of which—

I was mixed with the same volume of *Cl. welchii* broth culture filtered through a Gradocol membrane APD 750 mμ.

II and III with the same volume of saline.

I and II were incubated at 35° C. for 60 minutes; III was placed at 0° C. until the titration was performed. For control, a sample of *Cl. welchii* filtrate and a sample of virus suspension were incubated at 35° C. for 60 minutes. The haemagglutination titre of each suspension was then determined (Table VI).

The titre values have been corrected for volume changes so that all the values in Table IV are directly comparable.

TABLE VI.—*Release of Haemagglutinins from the Complex Virus-Inhibitor by Treatment with a Filtrate of* Cl. welchii.

		Log agglutination titre.*
I.	Virus + inhibitor + *Cl. welchii* filtrate, 35° C. 60 min.	2·88
II.	„ + „ + saline, 35° C. 60 min.	0·98
III.	„ + „ + „ 0° C.	0·83
	„ 35° C. 60 min.	4·03
	„ not incubated	4·04
	Cl. welchii filtrate, 35° C. 60 min.	1·73
	Supernatant after centrifugation of virus + inhibitor	1·18
	„ „ washing the deposit with saline	0·35

* All titres in the table are corrected for volume changes so that they may be compared directly.

A comparison between titres II and III shows that the short incubation at 35° C. did not significantly increase the haemagglutination titre. Probably the virus concentrate used in the experiment had lost most of its IDC. On the other hand, after incubation in the presence of *Cl. welchii* filtrate the haemagglutination titre exceeded by one log unit the haemagglutination capacity of the filtrate itself, indicating a release of virus haemagglutinins.

That the same filtrate was able to inactivate the inhibitor, also in the absence of virus, was demonstrated by a complementary test summarized in Table VII.

Heating to 65° C. for 30 minutes destroyed the capacity of the *Cl. welchii* filtrate to inactivate the inhibitor.

The activity of the inhibitor in vivo.

In the following experiment the activity of the inhibitor was examined *in vivo*.

Eleven litres of clarified normal allantoic fluid containing merthiolate 1/10,000 were diluted to double the volume with 0·1 M phosphate buffer pH 7·0 also containing merthiolate 1/10,000. With the aid of ultrafiltration (Seibert, 1928) the suspension was concentrated to approximately 1 litre, washed on the filter with 5·5 litres of buffer solution containing 10 units of penicillin and 50 μg. of streptomycin per ml., and finally further concentrated to about 1/500 (20 ml.) of the original volume. The operation was carried out at 4° C.

Two volumes of this brown, thick, very viscous material were mixed with 1 volume of a highly infective allantoic fluid, diluted 10^{-6} in 0·1 M phosphate buffer pH 7·0 containing 500 units of penicillin and 1 mg. of streptomycin per ml. The mixture was allowed to stand at 4° C. for 45 minutes, whereupon 0·3 ml. amounts were injected into the allantoic sac of 17 eggs. In two control series, each containing 17 eggs, the virus and inhibitor components respectively of the inoculum were replaced by the same volume of phosphate buffer with penicillin and streptomycin. The eggs were incubated at 35° C.

The inhibitor concentrate proved to be highly toxic to the embryos. Of the embryos inoculated with the concentrate, all, excepting 3 in each series, were dead after 20 hours, and after 43 hours all were dead. In the control series without inhibitor concentrate all the embryos survived. Part of the allantoic fluid was collected from the surviving embryos after 20·5 hours, and after 43 hours the residual fluids were removed from these eggs. All the suspensions were

TABLE VII.—*Decrease in the Inhibitory Activity of Normal Allantoic Fluid by Incubation with Small Amounts of a Filtrate of* Cl. welchii.

Material added to the normal allantoic fluid		*Clostridium welchii* filtrate.	Filtrate previously heated to 65° C. 30 min.	Control without filtrate.
Residual inhibitory activity of normal allantoic fluid incubated with the *Cl. welchii* filtrate.*	Concentration of the filtrate	1/100.	1/100.	
	Hours of incubation 0	1·66	1·96	1·81
	Hours of incubation 4	1·36	..	..
	Hours of incubation 20	0·91	1·81	1·96

* Normal allantoic fluid was incubated with the filtrate at 35° C. Samples were removed after different periods of time and tested for residual inhibitory activity after heating at 65° C. for 30 minutes. Titre value of the saline control of the test virus 4·09.

Haemagglutination titre of the *Cl. welchii* filtrate 1·88. Effect of normal allantoic fluid on the titre, expressed as inhibitory activity 1·20.

TABLE VIII.—*Influence on Virus Reproduction of Mixing Virus with Inhibitor Concentrate before Inoculation.*

Allantoic fluids harvested after—	Titres of allantoic fluids from embryos inoculated with— Inhibitor + virus: Haemagglutination titre.	Inhibitor + virus: Inhibitory activity.*	Inhibitor + virus: 50 per cent chick embryo infectivity end-point.**	Buffer solution + virus: Haemagglutination titre.	Buffer solution + virus: Inhibitory activity.*	Buffer solution + virus: 50 per cent chick embryo infectivity end-point.**	Inhibitor + buffer solution: Inhibitory activity.*
20·5 hrs.	< 0·30	2·95	2·5	< 0·60	1·50	6·0	2·80
43 hrs.	< 0·60	2·65	6·8	4·39	0·60	7·8	3·10

* Inhibitory activity of the heat-inactivated test material (boiled and immediately cooled). Titre value of the saline control of the test virus 3·78.

** 3 eggs were used in each group.

sterile. The 50 per cent chick embryo infectivity end-point and the haemagglutination titre were determined as well as the inhibitory activity after boiling of the suspensions (Table VIII). Table VIII shows that influenza virus can multiply in the allantoic sac even if inoculated mixed with a large amount of inhibitor.

DISCUSSION.

Small quantities of allantoic fluid infected with the PR8 and Lee strains of influenza virus and mumps virus respectively have the capacity, when incubated at 35° C. with normal allantoic fluid, to destroy gradually the inhibitory activity. After sufficient destruction of the inhibitor haemagglutinins appear in the previously non-haemagglutinating mixture. This phenomenon is analogous to the elution of virus from red cells and from the cells of the respiratory tract (Hirst, 1942, 1943). It also agrees with the observations reported quite recently by Burnet and co-workers (1948) and Hirst (1948) that virus under similar conditions inactivates the inhibitive capacity of normal serum.

As virus purified by centrifugation retains this capacity to destroy the inhibitor in normal allantoic fluid, as well as the inhibitor in normal serum (Hirst, 1948), it is probable that the virus particles or substances absorbed to them take part in the inactivation process. This does not necessarily imply that the virus particles have the character of a complete enzyme system. It is evident, however, that they, in some way, activate the inhibitors and the receptor substance of the red cells preparative to their destruction.

Whether a hypothetical " coenzyme " of the inactivating system is to be found in the virus particle or in the corpuscular elements carrying the receptors or inhibitory activity must be left an open question. According to Volkert and Horsfall (1947) pneumonia virus of mice, fixed to cell receptors, is released only in the presence of some complementary substance from normal lung tissue.

Through cautious treatment with formalin it has been found possible to destroy entirely the infectivity of the virus, with little or no effect on its capacity to inactivate the inhibitor and to haemagglutinate. After heating at 56° C. for 30 minutes the IDC is insignificant, while the virus still is able to combine with the inhibitor and to haemagglutinate. Virus, thus treated, is more sensitive to the inhibitor. This phenomenon is in conformity with the observations of Francis (1947), Burnet (1948) and Hirst (1948) in respect to the serum inhibitor. As Burnet and Hirst have pointed out, such a result is to be expected if the power of the virus to destroy the inhibitor be inactivated.

The relative sensitivity to heat treatment of the inhibitor-destroying capacity rendered possible an experimental arrangement whereby a quantitative study of the virus-inhibitor interaction could be carried out. It became apparent, as previously mentioned (Svedmyr, 1947*b*, 1948), that the addition of active virus to a normal allantoic fluid interfered with the inhibitory activity even when the mixture was immediately heated—that is to say, without previous incubation. A quantitative analysis under such experimental conditions revealed that on the addition of increasing amounts of active virus, a steep fall in the inhibitory activity occurs. When the virus concentration reaches a certain limit, free haemagglutinins appear, and if the virus content is further increased, the inhibitory activity approaches a low but constant level. Even in pure infective allantoic fluids an inconsiderable residue of inhibitory activity is to be found. This

residue, judging from certain preliminary tests, can presumably be due to non-specifically inhibiting, probably dialysable factors which remain in the supernatant after centrifugation at 27,000 r.p.m. for 20 minutes.

From the curves in Fig. 1 can be seen that within a certain concentration interval, free haemagglutinating virus and free inhibitor can be simultaneously demonstrated. This circumstance coincides with the assumption that the virus-inhibitor interaction has the character of an adsorption reaction.

These observations may seem to be in opposition to certain results in one of Hirst's quite recently published papers (Hirst, 1948), where he states that he achieves no destruction of the inhibitor in normal serum if immediately after the addition of the virus to the serum he heat-inactivates the mixture. The explanation may be that Hirst employed so small doses of the virus that the effect on the inhibitor was not demonstrable.

The non-infective virus modification described by von Magnus (1947*a*, 1947*b*), Gard and von Magnus (1947), and Gard, von Magnus and Svedmyr (1947), which appears at serial passages of undiluted virus infected fluids, seems to have a capacity to inactivate the inhibitor, which to no appreciable extent differs from that of infective virus. On the assumption that the virus-inhibitor reaction reflects the course of the reaction *in vivo* between virus and the receptors of the susceptible cells, the loss of infectivity in this case can obviously not be assigned to the disappearance of the capacity to break down the receptor substance of the cells.

The report of Burnet and co-workers (1946) that certain bacterial enzymes were able to destroy red cell receptors suggested a test of the effect of a *Cl. welchii* filtrate on the inhibitor. This was found, even when in small doses incubated at 35° C. with normal allantoic fluid, to destroy the inhibitor, and, moreover, it had the capacity to release haemagglutinins from the virus-inhibitor complex. The result agrees with the recent report of Burnet and co-workers (1947) that a similar enzyme from *Vibrio cholerae* inactivates the inhibitor in normal serum. Of particular interest is Stone's statement (1947) that this enzyme can destroy the cell receptors in the allantoic sac.

If the inhibitor acts through combination with the virus particles, the release of which is possible only after destruction of the inhibitor, it should be possible to trace its activity also *in vivo*. If a concentrate of the inhibitor be mixed with virus and inoculated into the allantoic cavity, this should accordingly cause a retardation of virus multiplication, as virus only gradually would be released and able to combine with the cell receptors. On the other hand, the inhibitor should not be able to prevent but only retard virus multiplication.

The result of such an experiment revealed, as was expected, that virus could multiply in the embryo even if inoculated together with a large amount of the inhibitor. The effect on the rate of the virus multiplication, as demonstrated in Table VIII, cannot be considered significant on account of the small number of embryos surviving. On the other hand, it must also be borne in mind that the toxic effect of the inhibitor concentrate in itself might very well affect unspecifically the rate of virus multiplication.

SUMMARY.

Small quantities of active influenza and mumps viruses have the capacity, when incubated at 35° C. with normal allantoic fluid, to destroy gradually the

inhibitory activity. By means of treatment with formalin and heat, it is possible successively to destroy the capacity of the virus to infect, inactivate the inhibitor, combine with the inhibitor and haemagglutinate.

A quantitative study of the virus-inhibitor interaction has been performed and the result is discussed.

It is possible with the aid of *Cl. welchii* filtrate to destroy the inhibitor and release the haemagglutinins from the virus-inhibitor complex.

Virus can multiply in the embryo even if a large dose of inhibitor is added to the inoculum.

I am indebted to Dr. Sven Gard for valuable advice and criticism throughout this work.

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THE PATHOGENIC EFFECT OF PHTHIOIC ACID AND ITS SYNTHETIC ANALOGUES.

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SABIN, Doan and Forkner (1930) and Sabin, Smithburn and Thomas (1935) made the first systematic attempts to produce experimental lesions in animals with chemical substances fractionated from tubercle bacilli. They stated that only the substances from the lipoid fractions produce tuberculous lesions. Anderson's phosphatide A. 3 was found by these authors the most active of all fractions in forming epithelioid and giant cells. Examination of its constituent fatty acids indicated that the optically active phthioic acid had a similar though less marked effect, while the optically inactive tuberculostearic acid was relatively inert. Gerstl and Tennant (1943) and some French investigators, including Buu-Hoi and Ratsimamanga (1943), have obtained granulomatous lesions after injecting synthetically produced long chain fatty acids into animals, but the pathological changes are not described in sufficient detail by the latter authors. Preliminary tests with two acids of the trisubstituted acetic acid type produced by Stenhagen and Stalberg (1941) and supposed to constitute the phthioic acid molecule did not show specific tissue reaction; in addition, Polgar and Robinson (1943) have proved that the formulation of phthioic acid as a trisubstituted acetic acid was incorrect.

Arguments against Sabin's views were presented by a number of authors. Thus Boissevain and Ryder (1931) objected that the method of purification used by Anderson for the lipids from the tubercle bacillus did not ensure freedom from bacillary debris, and they claimed that the lipoid fraction freed from bacillary particles did not provoke the tissue reaction described by Sabin as following injection of Anderson's phosphatide. These authors state that the reaction of the suggested phthioic acid must be classified as a foreign-body reaction. Similar critical views are expressed by other investigators (Bloch, 1936*a*, *b*), who maintained that the specific activity of the phosphatide is not due to the presence of phthioic acid, but rather to the structure of the phosphatide molecule itself.

As the problem of the pathogenic effect of the fatty acids from the tubercle bacillus is still controversial, we welcomed the opportunity given to the Therapeutic Research Corporation by Sir Robert Robinson to investigate the pathogenic effect of a number of synthetic acids closely related chemically to phthioic acid (Polgar and Robinson, 1945). The acids were synthetized in the Dyson Perrins

Laboratory at Oxford by Dr. N. Polgar, partly in collaboration with Mr. S. David and Dr. E. Seijo. It should be stated that phthioic acid is the only constituent of the phosphatide from tubercle bacilli that Anderson (1929), and afterwards Sabin (1941), found to be capable of producing lesions resembling those of tuberculosis (Anderson and Chargaff, 1929*a*, *b*). Anderson suggested for this the formula $C_{26}H_{52}O_2$. According to Anderson it occurs in all of the three major lipoid fractions of the tubercle bacilli.

We studied the effect of injecting the synthetic fatty acids into experimental animals to establish (*a*) the acute toxicity, (*b*) the lesion producing activity and (*c*) two other biological properties, namely, allergic properties and hapten-like character. For convenience we have labelled a compound as active if intraperitoneal injections of 100 mg. or less caused granuloma-like lesions and inactive when no lesions were found. It should be added that this classification is a relative one, for some substances inactive at 100 mg. might show a few lesions at 200 mg. or more. This report covers the histopathology of the granulomatous lesions produced by the substances; the immunological study of fatty acid complexes (polysaccharides and protein conjugates) will be reported later.

Acute toxicity was determined by intraperitoneal injections into mice and guinea-pigs. In a few instances the substances were injected into the skin to observe the local reaction. The use of suitable solvents was investigated and arachis oil and olive oil were used at the beginning, but we had to abandon these because in some animals there was a reaction to arachis oil injected into the abdominal cavity (visible as inflammation of the omentum, enlargement of lymph glands and free round pearl-like bodies in the peritoneal cavity). Propyleneglycol was also used to dissolve the acids, but the procedure finally adopted for the injection was to dissolve them in absolute ethyl alcohol and then dilute in saline to give a 10 per cent or 20 per cent suspension.

The following is a list of the methyl substituted long-chain acids tested, with an indication of their activity.

T.R.C. No.	*Compound.*	*Activity.*
298	2:2 Dimethylstearic acid	Inactive at 100 mg. dose.
320	2:12:15-Trimethyldocosanoic acid	Active at 50 mg.
337	3:6:9- ,, ,,	Slightly active at 100 mg.
270	3:12:15- ,, ,,	Active at 25 mg.
324	3:12:15-Trimethyl-Δ^{12}-docosanoic acid	Active at 100 mg. Slightly active at 50 mg.
289	3:13:16-Trimethyldocosanoic acid	Slightly active at 50 mg.
255	13:17:21-Trimethyldocosanoic acid	Inactive at 200 mg.
256	2:13:17:21-Tetramethyldocosanoic acid	Inactive at 200 mg.
292	3-Methyltricosanoic acid	Inactive at 100 mg.
303	3:13-Dimethyltricosanoic acid	Active at 50 mg.
288	13:16-Dimethyltricosanoic acid	Inactive at 100 mg.
275	3:13:19-Trimethyltricosanoic acid	Active at 100 mg.
274	3:13:19-Trimethyl-$\Delta^{13,19}$-tricosadienoic acid	Active at 100 mg.
200	4:13:16-Trimethyltricosanoic acid	Active at 50 mg.
257	2:13-Dimethylpentacosanoic acid	Inactive at 200 mg.
282	Crude d-rotatory acid from human tubercle bacilli	Slightly active at 100 mg.

A short account of the chemical structure of these fatty acids follows: it is based on data supplied by Dr. Polgar.

13:17:21-*trimethyldocosanoic acid* (255) has a sequence of three methylene groups between the methyl branches (isoprene units), with one of the methyls at the far end of the chain (C-21).

2:13:17:21-*tetramethyldocosanoic acid* (256) bears an additional methyl in the α-position. In the 2:13-dimethylpentacosanoic acid (257) the C-17 and C-21 methyls of the foregoing acid are absent.

3:13:19-*trimethyltricosanoic acid* (275) has the probable formula advanced for *phthioic acid* and bears a methyl substituent in the β position.

3:13:19-*trimethyl*-$\Delta^{13:19}$-*tricosadienoic acid* (274) differs from 3:13:19-*trimethyltricosanoic acid* (275) only by having olefinic linkages adjoining two of the methyl branches. Since the olefinic linkages were produced by dehydration of tertiary carbinols, they may have been formed in the 12:13 or 13:14 and in the 18:19 or 19:20 positions respectively; also geometrical (cis and trans) isomers may be expected to arise.

4:13:16-*trimethyltricosanoic acid* (290) was prepared from 3:12:15-*trimethyldocosanoic acid* (270) by chain-lengthening, thus removing the methyl nearest to the carboxyl from the β to the γ position. Though it probably does not contain substantial amounts of 3:12:15-*trimethyldocosanoic acid* (270), the presence of this cannot be excluded with certainty.

All these acids with the exception of 2:2-dimethyl stearic acid contain asymmetric carbon atoms and will be mixtures of optical isomers.

Preliminary acute toxicity tests in mice and guinea-pigs have shown that the substances have, with one exception, no acute toxic effect in quantities of 1000 mg. per 1 kg. mouse or 800 mg. per 1 kg. guinea-pig.

EXPLANATION OF PLATES.

FIG. 1.—Patchy fibrinoid necrosis of the omental fat tissue with moderate cellular response around necrotic area. Guinea-pig. T.R.C. 254. 0·5 ml. Killed after 7 days. Omentum H. & E.: × 125.

FIG. 2.—Clusters of acid-fast corpuscular substance forming the centres of histiocytic nodules, in the periphery. Epithelioid cells and occasional giant cells of the Langhans type. Guinea-pig. Smegma antigen 100 mg. Killed after 12 days. Omentum H. & E.: × 200.

FIG. 3.—A. Edge of necrotic nodule to show necrosis and granulomatous tissue of periphery. Guinea-pig. Liver. T.R.C. 270. 50 mg. Killed after 16 days. H. & E.: × 80.

FIG. 4.—A.1. Edge of necrotic nodule to show necrosis and granulomatous tissue of periphery. Guinea-pig. Liver. T.R.C. 270. 50 mg. Killed after 16 days. H. & E.: × 175

FIG. 5.—Necrotic foci to show (1) necrosis; (2) histiocytic proliferation and (3) beginning encapsulation. Guinea-pig. T.R.C. 270. 0·1 ml. undiluted. Killed after 17 days. Omentum H. & E.: × 90.

FIG. 6.—Massive necrosis of omental fat attached to intestinal wall, with histiocytic cell infiltration and beginning formation of nodule in the periphery. Guinea-pig. T.R.C. 254. 1 ml. undiluted. Killed after 30 days. Omentum H. & E.: × 60.

FIG. 7.—Giant cells in omental fat. Guinea-pig. T.R.C. 254. 0·1 ml. undiluted. Killed after 30 days. Omentum H. & E.: × 220.

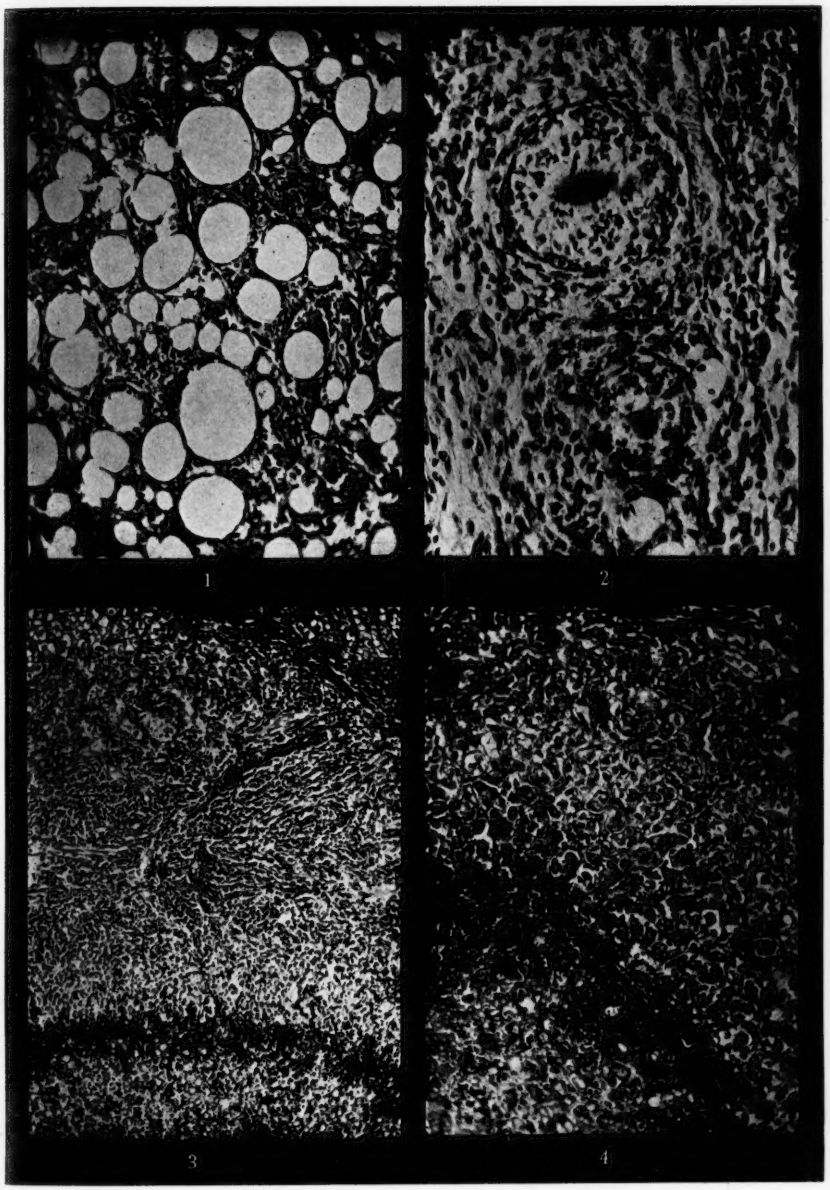
FIG. 8.—Fibrosing nodules in the later stages. Lymph gland. Guinea-pig. T.R.C. 254. 0·1 ml. undiluted. Killed after 30 days. Omentum H & E: × 110.

FIG. 9.—Necrosis with calcified patches. Omentum. Guinea-pig. T.R.C. 270. 0·1 ml. undiluted. Killed after 35 days. Omentum H. & E.: × 65.

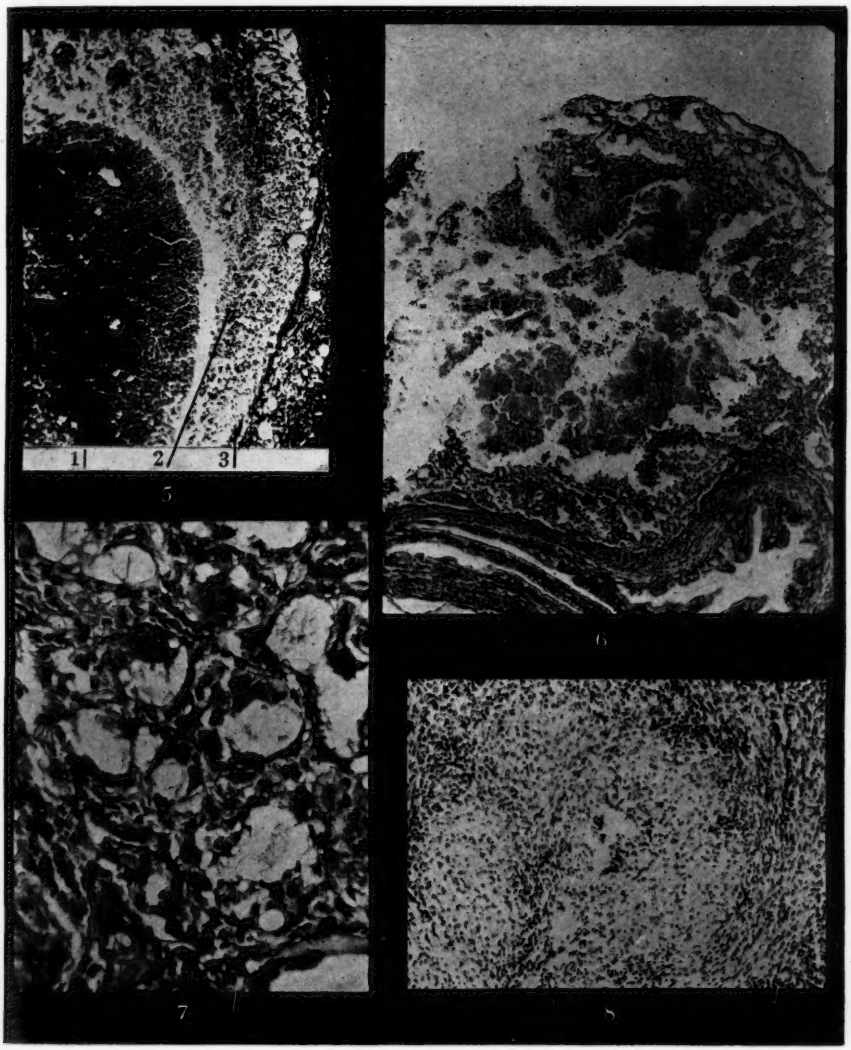
FIG. 10.—Granuloma consisting of macrophages containing fat and giant cells. Guinea-pig. T.R.C. 254. 0·1 ml. undiluted. Killed after 47 days. Omentum H. & E.: × 55.

FIG. 11.—B. Formation of histiocytic and giant cell nodules in the omentum. Guinea-pig. T.R.C. 254. 0·5 ml. Killed after 47 days. Omentum H. & E.: × 110.

FIG. 12.—B.1. High power magnification of histiocytic and giant cell granuloma. Guinea-pig. T.R.C. 254. 0·5 ml. Killed after 47 days. Omentum H. & E.: × 250.



Ungar, Coulthard and Dickinson.



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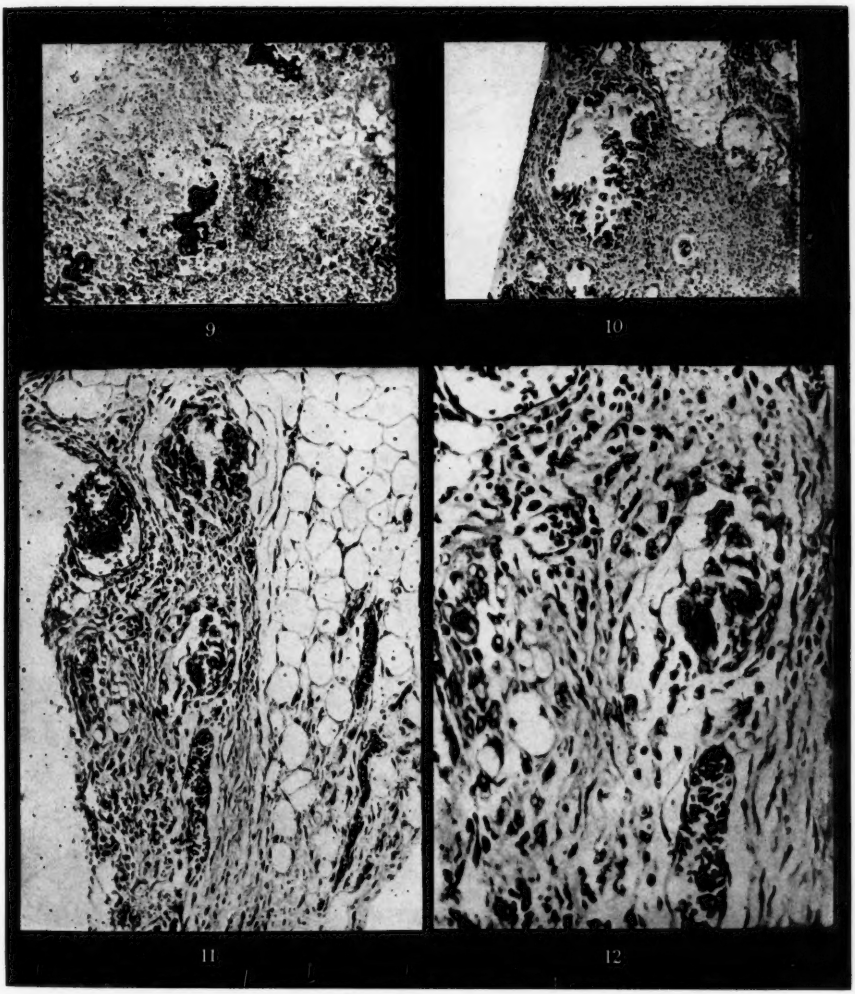

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11 12

Ungar, Coulthard and Dickinson.

The pathogenic properties of the fatty acids were studied in guinea-pigs. The substances were injected intraperitoneally into groups of 4 to 8 guinea-pigs (according to the amount of compound available) in doses of 10, 25, 50, 100 and 200 mg. (in a few instances). At regular intervals (3, 6, 8, 14, 28 days, 6 weeks and 3 months) guinea-pigs from each group were killed and postmortems performed, specimens being taken for histological examination.

Slight anatomical lesions were seen in guinea-pigs after 10 mg. injection with the active compound T.R.C. 270, but lesions followed the injection of 25 mg., and more markedly after 50 mg. or more. We give below some of the postmortem findings, representative of the many recorded.

Eight days after the intraperitoneal injection of 25 mg. of T.R.C. 270 a hard waxy deposit on the spleen and omentum, perisplenitis, enlarged mesenteric lymph glands and waxy deposits on the liver were seen.

Fourteen days.—White round nodules 4–5 mm. in diameter fixed on the peritoneal wall; omentum retracted towards the spleen, showing nodules of cheesy appearance ; pre-aortic glands enlarged, small nodules in the liver, spleen ; occasional small nodules in the lungs.

Twenty-eight days.—Occasional soft round whitish bodies 2–8 mm. in diameter in the abdominal cavity (mostly with Substance No. 254). Nodules of cheesy appearance in the indurated inflamed omentum ; omentum adherent to spleen and left kidney ; spleen enlarged ; perisplenitis ; mesenteric lymph glands enlarged ; pre-aortic glands enlarged ; liver adherent to the diaphragm ; nodules on the diaphragm ; in some cases adhesions of mesentery to peritoneal serosa ; small nodules in the lungs ; tracheo-bronchial glands enlarged.

Three months.—Omentum thickened, fibrotic, adherent to spleen, which showed fibrotic changes in the capsule. Portal glands enlarged, occasionally showing a small central caseous mass. Liver showing a few small nodules. Mesenteric glands enlarged and occasional enlargement of peritracheal glands.

One of the more active substances, 3:12:15-trimethyldocosanoic acid (No. 270), was tested for its effect on the skin. For this purpose 10 and 25 mg. in alcohol-saline were injected intradermally into guinea-pigs. A local reaction was visible after 24 hours in the form of erythema and central induration, which gave rise in the next 3 days to a marked nodule in the skin and caused in some guinea-pigs the breakdown of epidermis and discharge. These nodules persisted for 10 to 14 days and gradually disappeared.

The same substance, No. 270, was injected intravenously into guinea-pigs in 50 mg. doses without apparent ill effects. Guinea-pigs killed after 8–12 days did not show visible lesions macroscopically, except that in two guinea-pigs we noticed a few small gelatinous nodules in the lungs.

The sections of organs of guinea-pigs injected with the active compounds showed characteristic histological changes, most marked in the omentum and the serosae of abdominal organs, notably of lymphatic glands and spleen.

In the early stages 3 days after the injection small areas of patchy fibrinoid necrosis were seen in the omentum, occupying the stroma in the fat tissue. In addition there were large necrotic areas, some ill, others well defined, and mostly attached to organ capsules. They were separated from neighbouring structures by flakes of fibrin membrane or by omental fat tissue.

After 6 to 8 days multiple, occasionally large abscesses in the omentum were seen with collection of mononuclear cells (macrophages) beginning in the periphery. Many of these cells had assumed a distinct foamy appearance, obviously

due to absorption of fatty substances. In some instances patchy fibrinoid necrosis was predominant, the omental fat still being recognizable, as described at the three days' stage. There was also polymorph cell immigration into the necrotic patches.

Later, after 10 to 14 days, in addition to the abscesses, areas of necrosis and peripheral collection of foamy macrophages, a few syncytia engulfing disintegrating fat cells were seen and also the beginning of fibrous encapsulation in the periphery. Occasionally in the periphery of abscesses a nodular arrangement of macrophages and syncytia became discernible.

Skin : Two types of changes were visible after intracutaneous injection of fatty acids :

(*a*) infiltration with leucocytes (disintegrating) and fibrinoid necrosis in the subcutaneous tissue ;

(*b*) small granulomata in the subcutis consisting of mononuclear cells.

After intracardial injections some lung abscesses developed with peripheral foam cells collecting in atelectatic lung tissue.

17–19 *days*.—Large areas of fat necrosis in the omentum with patchy central calcification. Some collection of mononuclear, occasionally almost epithelioid cells at periphery. Sub-capsular areas of the liver containing well defined scars and infarcts surrounded by nodules of foamy macrophages, attached to the capsule.

27–35 *days*.—In the omentum encapsulated abscesses or areas of necrosis with patchy calcification, also foam-cell nodules and foreign-body granulomata in the periphery. Nodules of foam cells with central necrotic patch, as seen in subcapsular areas of liver and spleen and lymph gland, occasionally assuming a resemblance to tubercles with some of the cells containing nuclei poor in chromatin and reminiscent of epithelioid cells and giant cells of the Langhans type. Also giant cells of the foreign-body type and syncytia apparently formed by the confluence of macrophages.

50–60 *days*.—In the omentum granulomata, mostly of the foreign-body type, surrounding large areas of necrosis as well as small necrotic patches, the necrotic areas having fibrous capsules and calcified patches, often in the form of antlers—not unlike those seen in calcifying tubercular foci. Spleen showing a cellular pulp and necrotic areas, some already encapsulated, others well demarcated by zonal collection of necrotic polymorphs. Liver at this stage showing a number of nodules consisting of hyaline collagen (scars), and between them sheets of foamy macrophages mixed with lymphocytes, the latter increasing in number towards the periphery, where they formed a line of demarcation.

A summary of the changes will be given in the discussion. It may be said, however, that the picture obtained seemed to correspond in some respects to the description by Sabin, Doan and Forkner (1930) of the effects of fatty acids isolated from tubercle bacilli. The type of fatty acid used seemed to account for differences in the intensity and appearance of the lesions. For example. substance T.R.C. 290 elicited very little cellular activity in the periphery of the small encapsulated homogeneously necrotic foci.

The lesions produced by phthioic acid analogues differed from those produced by waxy substances. We injected guinea-pigs intraperitoneally with a suspension of beeswax in arachis oil and with myrosin, a fraction extracted from beeswax : necrosis did not occur with either. In the omentum a foreign-body

granuloma developed ; it was cellular and devoid of central necrotic changes and leucocytic debris, which usually occur in the phthioic acid lesions.

To compare the activity of the synthetic with that of the natural fatty acids a crude fatty acid (isolated from a human type bacillus), T.R.C. 282, and also ground autoclaved tubercle bacilli (human type) and smegma bacilli, were injected intraperitoneally into guinea-pigs in identical doses (25, 50, 100 mg.). The fatty acid T.R.C. 282 showed only weak activity at 50 mg. and gave rise to lesions similar to those produced by the active synthetic compound No. 270, namely, necrotic changes in the omentum, enlargement of the mesenteric lymph glands and spleen, perisplenitis, nodules in the liver and on the diaphragm. (In addition to the crude fatty acids from the human type *Myc. tuberculosis* the crude fatty acid from *Myc. phlei* had been examined. Intraperitoneal injection of 25–50 mg. of the former crude substance produced changes detectable after 2–3 weeks, but no changes were seen 2 weeks after injection of 100 mg. of the latter.) The ground bacilli (human type and smegma bacilli), however, elicited classical tubercles at an early stage ; the response thus differed from that to phthioic acid analogues, in which foreign-body and foam cell granulomata predominated ; occasionally resemblance to genuine tubercles was obtained in the late stages. Tubercles with typical Langhans giant cells and epithelioid cells were visible in the omentum as early as 8 days after injection of the ground bacilli. The earliest change after 3 days was a diffuse histiocytic infiltration and only occasionally an area of fibrinoid necrosis, although later on larger abscesses were observed (for example, after 28 days). Ziehl-Neelsen stain revealed acid-fast material, chiefly granules, but also rods in excessive quantities.

A number of fatty acids were tested for the response of tuberculous guinea-pigs to their intradermal injection. No hypersensitive reaction was noticed ; the skin of the infected animals reacted to the same degree as that of the control guinea-pigs.

DISCUSSION.

The changes induced in guinea-pigs after injection of phthioic acid analogues were uniform in development and appearance. These were—

(1) At first patchy, then confluent necrosis followed by leucocytic immigration (abscess formation), and either softening or patchy calcification.

(2) Macrophages (mononuclear cells, histiocytes) appearing in the periphery at the six days' stage. They were large and distinctly foamy, indicating absorption of fatty substances.

(3) At the 10 to 14 days' stage macrophages arranged in nodules, some of which showed central necrosis (or an abscess). Some were "tuberculoid," others more like foreign body granulomata. Some contained syncytia or giant cells of the Langhans or else of the foreign-body type.

(4) At later stages abscesses or necrotic areas still present or else scars, sometimes surrounded by nodular granulomata. The changes developed at the site of injection, and there was evidence of lymphatic spread from the primary deposit (in the omentum and serous capsule of organs after intraperitoneal injection) into the periphery of some of the organs, where infarcts, areas of necrosis and granulomata were occasionally found. In the omentum itself there was first a fibrinoid infiltration of the septa

between the fat tissue, then necrosis became confluent and widespread, but not infrequently the original pattern of the fat tissue remained for a long time.

The changes elicited by the fatty acids were consistent with massive necrosis, abscess formation and granulomata due to administration of an irritant foreign body of lipoid character. In spite of this some of the changes were not without similarity to tubercular lesions, but differed from those elicited by the granular debris of human type or smegma bacilli, the latter being indistinguishable from genuine tubercular changes. The reason for this is the presence of corpuscular acid-fast material (granules and rods) in the inoculum, absent from suspensions of the fatty acids. It is at present difficult to correlate the pathogenic properties of the synthetic fatty acids with their chemical structure. It is possible that the position of the methyl groups in the chain may be an essential factor responsible for the biological activity of the molecule.

SUMMARY.

(1) A number of synthetic fatty acids, closely related to the naturally occurring phthioic acid, on injection intraperitoneally into guinea-pigs caused changes in the organs of the abdominal cavity, mainly in the omentum, lymph glands and spleen.

(2) The main characteristics of the lesions were necrosis, abscess formation and foam cell nodules, which may exhibit the features of a foreign body or a tuberculoid granuloma.

(3) The action of the synthetically produced fatty acids was compared with that of the naturally occurring phthioic acid and found to produce similar lesions in guinea-pigs.

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VACCINIA AND ECTROMELIA IN THE MOUSE.

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THE virus of infectious ectromelia can be inactivated with ultra-violet radiation; such inactive virus can evoke immunity, and can suppress the lethal effects of living virus inoculated intraperitoneally into mice at the same time. Another example of the "interference phenomenon" is thus in evidence (Andrewes and Elford, 1947). Burnet and Boake's (1946) finding of a close relationship between the viruses of vaccinia and ectromelia prompted us to investigate the properties of irradiated vaccinia virus and the ability of vaccinia to interfere with the effects of ectromelia in mice.

MATERIALS AND METHODS.

The strain of ectromelia we used was the Moscow strain obtained from Professor V. D. Soloviev, and described in our earlier paper. The vaccinia virus was propagated in rabbit testes or by rubbing into the shaved skin of a rabbit. In the latter case the virus was concentrated and purified by differential centrifugation by our colleague Dr. A. S. McFarlane. Inactivation by ultra-violet radiation was carried out as described earlier (Andrewes and Elford, 1947).

Immunization with irradiated vaccinia.

Ectromelia inactivated by ultra-violet radiation proved capable of provoking definite immunity in mice. Vaccinia, now shown to be closely related to ectromelia, is, however, generally believed to engender only a low grade immunity when inactivated by any means.

A washed virus-suspension produced lesions on intradermal inoculation of 0·1 c.c. into a rabbit's skin when diluted 10^{-8}. This was first diluted 1 : 50 in buffered saline (M/60 phosphate pH 7·4). Irradiation for 4″ under the conditions used earlier for ectromelia sufficed to inactivate it, as shown by intradermal tests on rabbits; treatment for 1·7″ did not inactivate. The 4″ vaccine was then given intraperitoneally to mice in 0·5 ml. doses; the mice were challenged with living ectromelia (1 in 1000 dilution) intraperitoneally as shown in Table I.

TABLE I.—*Immunizing Value of Irradiated Vaccinia.*

Vaccination course.	Two doses a week apart. Challenge 1 week later.	One dose. Challenge 1 week later.	One dose. Challenge 2 weeks later.	Unvaccinated controls.
Results of challenge.	SSSSSS	+++++S	+++++++	+++++++

\+ = death from ectromelia.
S = survived 3 weeks.

This result suggested that irradiated vaccinia had good immunizing value provided that two doses were given. Unexpectedly we found no references in the literature to tests of irradiated vaccinia. We therefore carried out tests of the immunizing potency of this vaccine in rabbits and guinea-pigs. In a first experiment 2 rabbits received 5 c.c. doses of U.V.R. vaccine intraperitoneally 11 days apart, and were challenged intradermally with graded doses of live virus 8 days after the second dose; in a second trial the two doses of 5 c.c. were given 6 days apart and the challenge was 6 days later. There was no conclusive evidence of immunization. Similar experiments were carried out on 5 guinea-pigs which received (first experiment) two doses of 3 c.c. intraperitoneally a week apart, or (second experiment) two doses of 5 c.c. subcutaneously a week apart. Challenge was made by injecting dilutions of vaccinia into the foot-pads and shaved skin of the belly. The results were irregular, but indicated some immunity against 1 to 10 minimal infective skin doses, i.e. about as much as was found by Bland (1932) in his trials of formolized vaccinia in guinea-pigs. The efficacy of the U.V.R. vaccinia vaccine against ectromelia in mice is, therefore, to be attributed to other causes than an intrinsic superiority of vaccine inactivated in that way.

Immunization with living vaccinia.

We readily confirmed the finding of Burnet and Boake (1946) that living vaccinia will protect mice against ectromelia. Complete protection against 100 lethal intraperitoneal doses of ectromelia was afforded by a single dose of concentrated vaccinia given intraperitoneally 14 days beforehand. When challenge with ectromelia was made into the foot-pad, deaths were prevented but local lesions were not. An immunizing effect of live vaccinia given intraperitoneally was apparent after 24 hours: on intraperitoneal challenge with ectromelia after that time, 5/6 mice survived; the only death was not due to ectromelia. This result raised the question as to whether we were dealing with immunization or interference, and we felt, as a result of our earlier work with ectromelia vaccines, that the problem was more likely to be clarified by study of the results of inoculations into foot-pads.

Vaccinia in the mouse's foot-pad.

Intraplantar injection into mice of vaccinia virus, either as washed concentrated dermal virus or suspension of infective testis, produced the following changes: Swelling of the affected foot appeared in 48 to 72 hours, and reached a maximum about the fifth day; it never became nearly as pronounced as with ectromelia. Superficial necrosis and desquamation of epithelium were apparent by the sixth day; during the second week, as the activity of the inflammation rapidly subsided, deformation of the foot was often apparent. Adjacent toes frequently became fused together, or toes stuck out at odd angles owing presumably to fibrotic contraction in deeper structures. Occasionally necrosis of a whole toe occurred, but the characteristic gangrene of ectromelia was absent. The whole process had passed its peak of activity at a time when lesions in mice inoculated at the same time with ectromelia were only beginning to appear.

A few attempts were made to transmit vaccinia serially in mice, removing pads, grinding them up, inoculating to pads of fresh mice; 2-day and 4-day

lesions were used, but in each case the lesions became rapidly less on passage. Attempts at adaptation were not, however, carried out intensively.

Interference tests in the foot-pad.

When a mixture was made of equal parts of undiluted living vaccinia and 1 in 1000 ectromelia, and this was injected into the foot-pads of 6 mice, typical ectromelia lesions developed, but only one mouse died, and that not from ectromelia. Of 6 controls receiving the same amount of ectromelia only, 5 died of ectromelia, and the sixth showed extensive lesions of ectromelia (ascites and fat necrosis) when killed 3 weeks later. When the vaccinia was injected into a foot-pad 24 hours ahead of 1:1000 ectromelia, again typical ectromelia lesions appeared, but none of 6 mice died. These results closely resembled those described earlier (Andrewes and Elford, 1947), when U.V.R.-inactivated ectromelia interfered with the action of living virus mixed with it and injected into the foot-pad. In both instances local lesions were relatively unaffected, but deaths were prevented. The next experiments suggested caution in concluding that a local interfering action could explain the two types of experiment equally.

Results of injecting vaccinia and ectromelia into opposite feet.

When the two living viruses were injected at the same time into opposite foot-pads, the results were the same as when they were mixed and injected together (see Table II).

TABLE II.—*Injection of Vaccinia and Ectromelia into Opposite Feet.*

Control (ectromelia alone).	Undiluted vaccinia into opposite foot.	1 in 10 vaccinia into opposite foot.
++++++	FFFFFF	+++FFF
++++F*	FFFFFF	FFFFFF

\+ = specific ectromelia death.
* = non-specific death.
F = ectromelia foot lesion and survived for 3 weeks.

Two other experiments gave similar results. In these tests a local "interfering" effect in the foot could not explain the life-sparing effect. It could alternatively be suggested that vaccinia, of which the lesions came up some days before those of ectromelia, might be very rapidly inducing an immunity which was effective in time to halt the march of the ectromelia. That this is the explanation is suggested by two facts. In mice receiving vaccinia in one foot and ectromelia in the other, the development of the ectromelia lesions was precocious as compared with those in mice receiving ectromelia only; they were evident after 5 days as against 6 days in the controls, and their evolution seemed a little more rapid. Such a result suggests that the vaccinia infection had altered the mouse's reactivity to the antigenically related ectromelia. Inoculation of herpes virus into the opposite pad failed to influence the outcome of ectromelia. In the next experiment, twelve mice were inoculated into the foot-pad with vaccinia and twelve with 1 in 1000 ectromelia. Pairs of mice were killed daily, and heart's blood and liver suspensions were tested for ectromelia by plantar inoculation into mice and for vaccinia by intradermal tests in

rabbits. Ectromelia was recovered from the heart-blood and liver after 5 days but not earlier, whereas vaccinia had reached the heart-blood after 3 days; it was not recovered from liver. Smith (1929) has shown that in rabbits anti-vaccinial antibodies can be demonstrated as early as the 2nd day after injection, and some experiments by one of us (Andrewes, 1931) suggested that active immunity to vaccinia might be present within 24 hours of injection of inoculation of virus and antiserum into opposite sides of a rabbit. In contrast to the results with living vaccinia, U.V.-inactivated ectromelia vaccine inoculated into one pad had no influence on deaths resulting from injection of living ectromelia in the other pad. In most ectromelia mice surviving as a result of concomitant vaccinia infection, macroscopic lesions of liver, spleen and other viscera were absent.

Effect of delaying vaccinia injection.

Five groups of 6 mice were injected into the left foot with ectromelia diluted 10^{-4}. One group received vaccinia into the right foot at the same time; to the others the vaccinia was given similarly after 1, 2, 3 and 4 days respectively. Table III shows the result.

TABLE III.—*Effect of Delaying Vaccinia Injection.*

	Vaccinia.			
Same day.	1 day later.	2 days later.	3 days later.	4 days later.
FFF	FFF	+++	+++	+++
FFF	FFF	GGG	+++	+++

+ = specific ectromelia death.
G = generalized ectromelia skin lesions, but survived 2 weeks.
F = ectromelia foot lesion but survived 2 weeks.

Clearly, delay of 24 hours in injecting vaccinia still permits it to save the lives of the mice, but at 48 hours the vaccinia is almost, and at 72 hours quite ineffective. A probable analogy may be drawn between this experiment and the results of vaccinating human beings shortly after exposure to smallpox.

Histological studies.

The lesions in ectromelia have been described in detail by Marchal (1930), by Greenwood, Hill, Topley and Wilson (1936), and recently, in a study of the natural transmission of the virus, Fenner (1947) has confirmed the widespread distribution of inclusion bodies. Since our primary object has been to determine whether protection by vaccinia is accompanied by any alteration in the character of the histological lesions, studies have, in the main, been confined to examination of the foot-pads and those organs which regularly present pathological changes—the spleen and the liver. The kidney in ectromelia occasionally shows naked-eye and microscopic abnormalities (Marchal, 1930; Fenner, 1947), so this organ was also included in the tissues regularly examined. We also studied the changes in the foot-pads, spleen, liver and kidney after vaccinial inoculation.

Ectromelia lesions.

The development and morphology of the inclusion bodies were fully described by Marchal, and it is only necessary to describe briefly the tissue changes in the

foot-pads. These do not become evident until the 6th day, the earliest sign being the development of localized foci of thickened squamous epithelium, brought about by enlargement and vacuolation of the cells of the *rete Malpighii*. Following the development of inclusion bodies, the nuclei become pyknotic, the cytoplasm disappears, and fluid-containing spaces are formed which coalesce with one another and rupture either into the dermis or externally. Oedema develops in the subcutaneous tissue around the 6th–8th day, and spreads outwards into the dermis and inwards, involving muscle and periosteum. Ulceration of the surface is rapidly followed by extensive infiltration of all the foot-pad structures by polymorphs; muscles, walls of arteries, tendons, joints and bone marrow are all involved. The arteritis is frequently followed by actual thrombosis, and gangrene of the extremities results, the extent depending upon the degree of vascular obstruction. Massive invasion by micro-organisms also takes place, and inclusion bodies are as a rule no longer demonstrable after this is established. The occurrence of liver and spleen necrosis is well known, and only a brief description is necessary. In the liver the process first affects small groups of cells within the lobules, the patches increasing in size and finally coalescing. In the spleen, necrosis is preceded by a tremendous hyperplasia of the primitive cells of the secondary nodules of the Malpighian bodies, formation of mature small lymphocytes being in abeyance. As in the liver, cell death occurs at first in small foci and quickly involves larger areas. As Fenner (1947) has pointed out, gross changes in the kidney occur irregularly, and in our series only one example has been obtained in which the renal architecture was markedly altered, and this was complicated by extensive bacterial invasion of the organ. A generalized capillary dilatation in the glomeruli was noted throughout, however, in the series, accompanied by increased prominence of the endothelial cells and occasional small foci of endothelial proliferation with mitoses. Small foci of round-cell infiltration in the cortex were sometimes found.

Vaccinial lesions.

In contrast to ectromelia, reaction to injections of vaccinia in the foot-pads occurs very quickly. Within 24 hours polymorphs have accumulated in considerable numbers in the subcutaneous tissue and between the muscle fibres, and numerous mitoses are seen in the germinal layers of the squamous epithelium, so that in 48 hours it has increased in thickness by several cell layers. Between the 3rd and 4th day, inclusion bodies of the nature of Guarnieri bodies appear in the *rete Malpighii*, the cells of which become increasingly vacuolated. Simultaneously a remarkable development of granules in the stratum granulosum takes place and hyperkeratinization is also apparent. Soon the nuclei of the affected patch become pyknotic, the cytoplasm disintegrates and the patches of degeneration are invaded by polymorphs. Ulceration quickly follows. Inclusion bodies are much less numerous than in ectromelia, and are confined to covering epithelium and hair follicles and are not found in the accessory skin glands as in ectromelia. The cellular reaction in the tissues of the foot-pad increases in intensity and spreads widely, but the walls of arteries never become affected. From the third day onwards fibrocytes enlarge, and soon a generalized but not uniform increase of connective-tissue cells is evident, and the subsequent formation of collagen is responsible for the fibrotic contraction and deformity of the foot structures seen at a later stage. Around the 12th day healing begins to take

place, and epithelium grows over the ulcerated areas. Secondary bacterial invasion is never so extensive as in ectromelia, healing is accompanied by fibrosis, and is not preceded by gangrene of the extremities as in ectromelia. No lesions have been found in the internal organs in the series examined.

Lesions of ectromelia modified by vaccinia.

Inoculation of vaccinia in the opposite foot-pad in no way alters the pathological changes due to ectromelia at the site of injection, although, as already stated, the process begins slightly earlier. One observes, however, a striking effect on the incidence of splenic and hepatic lesions, splenic necrosis being consistently absent, and liver necrosis absent or minimal. In the spleen, the demarcation between red and white pulp is almost obliterated by the hyperplasia of the lymphocyte precursors in the Malpighian bodies, but in contrast with the process when ectromelia alone is given, no necrosis takes place. In the liver, necrosis is generally absent; only occasionally the remains of a few necrotic cells are seen, although small accumulations of mononuclear cells in portal tracts and parenchyma are quite frequent, but as such foci are common in uninoculated animals, their significance is doubtful. In the kidney, dilatation of glomerular capillaries is quite striking, but is not accompanied by any increased cellularity.

DISCUSSION.

The experiments first described show that vaccinia inactivated with U.V. radiation can act as an immunizing agent, but suggest that it is not likely to be much more effective than other killed vaccinia vaccines.

Other experiments show that vaccinia can interfere with the lethal action of ectromelia injected at the same time. One cannot conclude, however, that this is a phenomenon altogether comparable with other instances of the "interference phenomenon" now so familiar amongst viruses. It is in fact very difficult to decide whether to interpret the results as due to interference or immunity. On the one hand one may visualize the pre-emption of cells by vaccinia, which thus inhibits the access or multiplication of the ectromelia; on the other hand, the more rapidly multiplying vaccinia may quickly engender an immunity against the slower ectromelia. Since vaccinia virus and its antibodies are likely to be well distributed in the body within a few days of injection, we have failed to devise a crucial experiment to decide between these alternatives. Nevertheless the facts presented incline us to believe that orthodox immunity rather than interference explains the results.

SUMMARY.

Vaccinia virus inactivated by ultra-violet irradiation will immunize mice against ectromelia. Such vaccines protect poorly against vaccinia in rabbits and guinea-pigs.

Vaccinia virus inoculated in a mixture with ectromelia into a mouse's foot-pad will interfere with the lethal outcome of the ectromelia; so, too, will vaccinia injected into the pad opposite to that receiving ectromelia. This sparing effect is possibly analogous to other examples of the "interference phenomenon," but can with equal or greater probability be explained as a result of immunity

rapidly induced by the vaccinia virus. The effect is still apparent when the vaccinia injection is delayed until 24 hours after the ectromelia.

Comparative study of the histological changes induced in the mouse's foot by ectromelia and vaccinia respectively supports other evidence that the vaccinial lesion evolves much the more rapidly.

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THE PREPARATION OF PURIFIED AND CONCENTRATED DIPHTHERIA TOXOID FROM A SEMI-SYNTHETIC MEDIUM.

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PART A.—TOXIN PRODUCTION.

MUELLER, in 1939, published a formula for the preparation of a semi-synthetic medium for the production of diphtheria toxin. In this medium he obtained a toxin titre of Lf 62 equal to 23 Lf per mg. of medium nitrogen. He stated that the optimal concentration of NaCl was about 0·5 per cent, and later Mueller and Johnson (1941) described methods for reducing the salt content of the casein hydrolysate. Using these methods Mueller and Miller (1941) obtained the very high toxin titre of Lf 100, equal to 37 Lf per mg. medium nitrogen.

For some time Mueller's first medium was used in these laboratories with consistent results. The only variation employed was to use a slightly lower concentration of casein nitrogen, namely 0·2–0·22 per cent. A toxin titre of Lf 62 was regularly obtained, but by further modification much higher yields of Lf per mg. of medium nitrogen have been achieved.

Experimental.

Unless otherwise stated *C. diphtheriae* P.W.8, strain "Toronto" was used, and cultures incubated at 34° C. for 7 days.

One of the earliest experiments carried out with Mueller's medium (1939) was to determine the limiting factors for growth. One factor proved to be the

source of nitrogen, namely the amino-acid mixture. This was done by adding to samples of a 10-day culture filtrate small quantities of the several components of the medium, reinoculating and noting growth. Furthermore, using impure natural amino-acids, the addition of small amounts of glycine, glutamic acid, aspartic acid and leucine, all permitted further growth. It was found, however, and this was of much greater importance, that if the concentration of hydrolyzed casein in the medium was much reduced, the addition of any of these amino-acids resulted in an increase of growth and toxin production. This did not occur if the medium contained initially 0·27 per cent casein nitrogen.

An alternative to removing the excess chloride from the neutralized casein hydrolysate was to use a lower concentration of casein-nitrogen than that recommended by Mueller with a corresponding lower chloride concentration, and to replace with prepared amino-acids those found to be deficient. As recorded above, four were found to be of some value. In preliminary experiments to explore this method, the toxin yields on media containing decreasing amounts of casein nitrogen were observed. The other constituents of the medium were kept constant, as described by Mueller (1939), except that for each level of nitrogen the optimal amounts of maltose and iron were determined. The following results were obtained :

Exp. 1826. 1/ix/43.

Conc. N_2 %.	Conc. Cl as NaCl %.	Lf.	Yield, Lf/mg. medium N_2.
0·05	(0·5)	30	60
0·10	0·55	50	50
0·135	0·73	62	46
0·20	1·1	62	32
0·25	1·4	52	20

These results showed : (1) that it is extravagant to use high concentrations of casein nitrogen in order to obtain moderately powerful toxins ; (2) that the yield of toxin-Lf/mg. of medium nitrogen increases as the N_2 and NaCl fall ; NaCl not weaker than 0·5 per cent.

Mueller's statement that 0·5 per cent NaCl is about optimal was confirmed as follows, using a sample of sulphuric acid hydrolyzed casein :

Exp. 1089. 28/viii/43.

Mixture No.	NaCl added to give— (%.)	Total N_2 used.	Lf titre.
A	0·2	0·19	52
B	0·4	0·19	60
C	0·5	0·19	65
D	0·6	0·19	62
E	0·8	0·19	57
F	1·0	0·19	52

At this stage Pappenheimer's work (Pappenheimer, 1936 ; Pappenheimer, Mueller and Cohen, 1937) on synthetic media was recalled. This work, it was

thought, would supply the identities of the amino-acids essential for growth and toxin production, and also *an approximation of the amounts required.* In the hope of obtaining some idea of the possible deficiencies in casein media at different concentrations, a comparison was made between Pappenheimer's figures and the amino-acid analysis of casein. In Table I is shown the amino-acid compo-

TABLE I.—*Comparative Amino-Acid Composition of Pappenheimer's Synthetic Medium and Diluted Hydrolyzed Casein.*

Amino-acid.	Pappenheimer's medium (1937).	Hydrolyzed casein		Deficiency at 0·8% casein
		1·6%.	0·8%.	
	(%.)	(%.)	(%.)	(%.)
Glycine	0·05	0·0064	0·0032	0·047
Valine	0·1	0·13	0·063	0·04
Methionine	0·02	0·0368	0·0184	0·002
Tyrosine	0·01	0·1	0·05	—
Leucine	0·05	0·16	0·08	—
Glutamic acid	0·5	0·348	0·174	0·326
Tryptophane*	0·01	—	—	—
Cystine†	0·02	(0·02)	(0·02)	—

* Tryptophane is very largely destroyed during the acid hydrolysis, and apparently is not essential for P.W.8, "Toronto."

† Cystine is added as a separate component in preparing the media.

sition of Pappenheimer, Mueller and Cohen's (1937) synthetic medium, and the concentrations of the same amino-acids in media containing the equivalent of 1·6 and 0·8 per cent casein. The high concentration of glutamic acid is an outstanding feature of the amino-acid composition of Pappenheimer's medium. The comparison showed that with approximately half strength Mueller's medium (0·8 per cent casein) at 0·135 per cent total nitrogen, the only serious deficiency was in glutamic acid.

Since it had already been found that using 0·1 per cent casein nitrogen (NaCl 0·5 per cent) the reasonable titre of Lf 50 could be obtained, it was decided to observe the effect of the addition of natural glutamic acid to media containing 0·1 per cent. casein nitrogen. The optimal amounts of added iron and maltose were determined for each total nitrogen level. The results obtained are shown in Table II. These results were of considerable interest and show a number of

TABLE II.—*Effect of the Addition of Glutamic Acid to Media containing 0·1 per cent Casein Nitrogen on the Toxin Titre.*
Exp. 1739. 13/v/43.

No.	Casein N_2.	Cl (as NaCl).	Glutamic acid added.	Total Nitrogen.	Lf.	Yield Lf/mg. N_2.
	(%.)	(%.)	(%.)	(%.)		
1	0·1	0·52	0·0	0·10	50	50
2	0·1	0·52	0·15	0·114	57	50
3	0·1	0·52	0·3	0·128	65	50
4	0·1	0·52	0·45	0·142	62	45
5	0·1	0·52	0·6	0·156	60	38

important points: (1) That the addition of small amounts of glutamic acid to media weak in hydrolyzed casein results in an appreciable increase in toxin titre; (2) the increase in titre itself represents a yield of 50 Lf/mg. nitrogen—equal to that obtained with the weak medium without the addition of the glutamic acid, namely the gain in titre is not obtained at the expense of the efficiency of the basal medium; (3) the addition of glutamic acid has an optimal concentration, which, if exceeded, decreases the toxin yield; (4) even when the concentration of casein nitrogen is reduced to 0·1 per cent, there is no deficiency of essential amino-acids, at least to produce a toxin titre of Lf 65.

In order to determinate whether the glutamic acid was essential in this concentration, or was merely a convenient source of nitrogen, the effect of additions of glycine to 0·1 per cent casein nitrogen media was observed; the results obtained are shown in Table III. These results are for all practical purposes

TABLE III.—*The Addition of Synthetic Glycine to Media containing 0·1 per cent Casein Nitrogen. Exp.* 1750. 26/v/43.

No.	Casein N_2. (%.)	Cl as NaCl.	Glycine added. (%.)	Toxin titre. (Lf.)	Yield. (Lf/mg. N_2.)
1	0·1	0·55	0·0	50	50
2	0·1	0·55	0·1	60	51
3	0·1	0·55	0·2	67	49
4	0·1	0·55	0·4	65	37
5	0·1	0·55	0·6	50	23

identical with those obtained when glutamic acid was used, and show that neither glutamic acid nor glycine is "essential" for the increased titre, but that both are convenient sources of nitrogen.

Other media were prepared containing rather greater concentrations of casein nitrogen, with a corresponding increase in the NaCl content. In brief, when varying amounts of glycine or glutamic acid were added, the titres increased to an optimum as previously experienced, but the peak yields (Lf/mg. medium nitrogen) fell as the concentration of casein nitrogen was increased, due no doubt to the inhibiting effect of the NaCl present. For example, a medium containing 0·135 per cent casein nitrogen with 0·7 per cent NaCl plus 0·1 per cent glycine and 0·25 per cent glutamic acid produced a toxin of Lf 80, thus giving a yield of 44 Lf/mg. nitrogen.

In view of the difficulty of obtaining large quantities of glutamic acid during the war, and the fact that synthetic glycine gave similar results, the routine medium was changed to one containing 0·1 per cent casein nitrogen (NaCl $\pm$ 0·55 per cent), plus 0·15 per cent glycine. The total nitrogen in this medium was therefore 0·128 per cent and a toxin titre of Lf 65 regularly obtained, which represents a yield of 50 Lf/mg. nitrogen.

Discussion.

The finding that the efficiency of a medium whose sole source of nitrogen is hydrolyzed casein increases as the concentration of nitrogen is decreased is one of considerable interest. It is known that excess iron and chloride are inhibitory to toxin production; in fact there are clear-cut optima for both. It may now be

concluded that in HCl hydrolyzed casein there exists a substance also inhibitory to toxin production since, when the concentration of NaCl is controlled, the yield (Lf/mg. medium nitrogen) increases as the nitrogen is reduced. Yet, if a dilute casein medium (i.e. 0·1 per cent nitrogen) be augmented with amino-acid nitrogen in the form of glycine or glutamic acid, there occurs a pronounced increase in toxin titre, with no reduction in the efficiency of the medium, up to the optimal amount for each amino-acid. Since it was found that excess of the amino-acids glycine and glutamic acid resulted in reduced toxin titres, the inhibitor may be one or more amino-acids, although some other substance may be responsible.

An interpretation of these findings is that in hydrolyzed casein there exists a deficiency of unessential nitrogen, relative to its content of essential amino-acids. When the available unessential nitrogen is augmented by the addition of glycine or glutamic acid, the surplus essential amino-acids are utilized.

Summary.

(1) The toxin yield—Lf per mg. of medium nitrogen—from a hydrolyzed casein semi-synthetic medium depends not only on the iron and NaCl concentration, but on the concentration of amino-acid nitrogen.

(2) This yield increases as the nitrogen in the medium is reduced—from 0·25 per cent down to 0·05 per cent nitrogen, iron and maltose optima being preserved, and NaCl not less than 0·5 per cent.

(3) The toxin titre in low casein nitrogen media (0·1 per cent casein nitrogen) is considerably augmented by the addition of small amounts of glycine or glutamic acid. There is an optimal concentration for either amino-acid to give the maximum gain in titre. The amount of additional amino nitrogen required to obtain maximal gain in titre is approximately the same for each of these two amino-acids.

(4) The increase in titre, obtained by adding the optimal amount of glycine or glutamic acid to the low nitrogen medium, itself represents the exceptionally high yield of 50 Lf/mg. amino-acid nitrogen.

(5) These findings and their possible interpretation are discussed.

PART B.—THE CONVERSION OF TOXIN TO TOXOID.

In the conversion of toxin to toxoid by the action of formalin, there are many factors operating. The following five were investigated in detail:

(1) Reaction, (2) temperature, (3) formalin concentration, (4) amino nitrogen content of the toxin, (5) redox potential.

(1) *Reaction (pH).*

It seemed evident that a knowledge of the stability of toxin and toxoid—derived from semi-synthetic media—at different pH levels would supply the information necessary for satisfactory control of pH during the toxoiding process. During this process there occurs a change in the ratio of toxin to toxoid, until no detectable toxin is present. Thus, if the optimal pH level for toxin is different from that for toxoid, it would follow that at first the best reaction should be one to favour stability of toxin, and later of toxoid.

Samples of fresh toxin were adjusted to different pH levels (glass-electrode pH metre) and placed in a 45° C. water-bath for an hour. They were then cooled,

their reaction checked, adjusted to pH 7 and their titres determined by the flocculation method. Samples of toxoid were similarly treated, and the results obtained shown in Table IV.

TABLE IV.—*The Influence of Reaction on Toxin and Toxoid when Heated to 45° C. for one hour. Exp.* 1816, 1817. 27/viii/43.

Toxin initially Lf 60, Kf 6
Toxoid initially Lf 60, Kf 18
} Flocculation tests carried out at pH 7·0.

Reaction. (pH.)	Toxin.		Toxoid.	
	Lf.	Kf.	Lf.	Kf.
5·0	10	—	55	24
6·0	30	18	57	20
7·0	57	9	60	21
8·0	57	10	57	23
9·0	57	10	57	31
10·0	51	37	20	—

These experiments showed that in both cases there is a pH *range* of stability for both toxin and toxoid.

Toxin is stable in respect of Lf and Kf (determined at pH 7) between pH 7 and 9. Toxoid is stable between pH 6 and 7·5. In general, toxoid is more stable on the acid side of neutrality, and toxin on the alkaline side. These findings are directly applicable to the process of toxoiding. Toxin may with safety be adjusted up to pH 9, if necessary, to take up the acidity of the formalin and acid formed by deamination. In keeping with the above argument it follows that the early stages of toxoiding require a slightly alkaline reaction and the later stages a slightly acid reaction.

(2) *Temperature.*

It is well known that toxin is far more readily destroyed by heat than is toxoid. The influence of relatively low temperatures on toxin does not appear to have received the attention it deserves, especially as this has a direct bearing not only on the process of toxoiding but on culture temperature.

A quantity of fresh toxin (toluene preserved) was adjusted to pH 7·6, and 50 ml. samples placed in bottles. These were incubated at temperatures between 5° C. and 39° C., titrated before incubation and again after 7 and 14 days. The result of this experiment (Table V) showed that up to a temperature of 30° C.

TABLE V.—*The Influence of Temperature on Diphtheria Toxin. Exp.* 2053. 14/iii/44.

Original Toxin Lf 62, Kf 8, pH 7·3. Reaction adjusted to pH 7·6. Preserved with toluene.

Temperature of incubation.		5° C.	20° C.	25° C.	27·5° C.	30° C.	34° C.	37·5° C.	39° C.
After 7 days	Lf	62	62	62	62	62	60	52	47
	Kf	10	10	10	10	10	11	12	15
After 14 days	Lf	62	62	62	62	62	57	52	47
	Kf	12	12	12	12	12	14	15	18

there occurred no change in toxin titre, or in Kf after 7 or 14 days, but at temperatures above 30° C. there was loss of antigen and increase in Kf, a change rapidly increasing with increase in temperature, and that diphtheria toxin has a fairly well-defined *critical temperature* (at pH 7·6), namely at about 30° C. The absence of loss in titre between the first and second week was unexpected.

A further experiment was carried out in which samples of a batch of toxoid were incubated at 30° C. at different reactions. The adverse effect of an alkaline reaction was confirmed, and the pH zone for minimal damage confirmed as lying between pH 6·0 and pH 7·0 (Table VI).

TABLE VI.—*The Influence of Reaction of Toxoid Stored at 30° C. for 7 days. Exp.* 2140. 16/vi/44.

Parent toxin originally Lf 60, Kf 9
Toxoid initially Lf 60, Kf 21
} Flocculation tests carried out at pH 7·0.

	Initial pH.	Final pH.	Lf.	Kf.
Reaction:—	4·5	4·9	42	55
	5·0	5·4	52	31
	5·5	5·8	57	24
	6·0	6·2	60	23
	7·0	7·0	60	23
	8·0	7·6	60	27
	9·0	8·7	60	35
	9·5	9·4	52	90
	10·0	9·9	5	—

The above findings would account for most of the antigen loss encountered when formolized toxins were immediately incubated at 37–39° C., amounting to about 25 per cent when freshly formolized toxins were incubated at 38–40° C., namely on the top shelf of a large incubator. This loss is avoidable by reducing the temperature to 30° C., but as would be expected a longer period of incubation is required—four weeks instead of three—to ensure complete atoxicity. In addition the increase in Kf (neutral mixture) for such toxoids is only three times that obtained with their parent toxins, and often only slightly greater than twice. When toxin-formalin mixtures were incubated at 38–39° C., the increase in Kf was often 5 or 6 times that of the parent toxin. The small reduction in Kf would seem to indicate a minimum of damage inflicted on the antigen. On the question of incubation temperatures, these results agree well with the practice of growing cultures for toxin production between 30 and 35° C.

(3) *Formalin concentration.*

(4) *Amino-nitrogen content of the toxin.*

Barr, Glenny, Pope and Linggood (1941) have published a table giving the optimal amount of formalin (40 per cent formaldehyde solution) to add to any sample of Pope and Linggood's (1939) "low" nitrogen digest medium-toxin. The amount of formalin required is correlated with the amino-nitrogen content of the toxin, and certain pH adjustments are recommended.

To obtain a similar guide for use with amino-acid media, of high or low amino-nitrogen content, the following experiment was carried out: A quantity of fresh toxin, prepared in low nitrogen medium (0·128 per cent N_2), was divided into six parts, and to five were added increasing amounts of the original concentrated stock solution of hydrolyzed casein. Final amino-nitrogen concentrations were between 0·5 N/10 and 1·0 N/10 amino-nitrogen. The amino-nitrogen values were obtained by using the usual Sorensen formol-titration technique. Each sample was titrated for its amino-nitrogen content and then divided into five parts; to each part different concentrations of formalin were added, giving in all 30 preparations. All samples were adjusted to pH 8·4 before the formalin was added. The samples were allowed to stand at room-temperature for 7 days, and then each examined for reaction, Lf and Kf, and their reactions adjusted to pH 7·4. The samples were then transferred to a 30° C. incubator, and left for four weeks. After the four weeks incubation they were again examined for Lf, Kf, reaction and toxicity—the latter by injecting 0·2 ml. intradermally into normal white guinea pigs.

The results obtained may be summarized as follows:

Initial AmN_2 N/10.	Minimum % formalin to detoxify.	Initial.		Final.		Antigen loss %.
		Lf.	Kf.	Lf.	Kf.	
0·49	0·325	60	8	57	13	5
0·63	0·35	59	8	57	13	3
0·70	0·40	58	8	55	13	5
0·82	0·425	56	8	52	14	7
0·92	0·45	55	8	52	15	6
0·99	0·475	54	8	51	15	6

It was subsequently found that since these data give *minimal* amounts of formalin, occasionally toxoids were not atoxic, in that guinea-pigs died of paralysis 25–30 days after receiving 5 ml. of the formol toxoid. An increase of 15 per cent of the amount of formalin used resulted in all samples being atoxic, and there occurred no adverse effect on the quality of the toxoid produced. These data may be reduced to the following simple formula:

Per cent formalin to add to toxin = 0·4 amino N_2 (N/10) + 0·2 per cent v/v, e.g. when the amino nitrogen titre is 0·5 N/10, the amount of formalin to add is 0·5 × 0·4 + 0·2 per cent v/v, or 0·4 per cent v/v.

This result confirmed the previous observations that, provided a temperature of 30° C. was not exceeded, toxoiding may be carried out with little loss of antigen and only small increase in Kf, and that the minimum amount of formalin required to detoxity does not necessarily give the smallest loss of antigen.

(5) *Redox potential.*

To observe whether aërobic or anaërobic incubation of formolized toxins had any effect on the quality of the final toxoid, samples of formolized toxin were placed in bottles in which one set was half filled and plugged with cotton-wool, and a mark made on the bottle to indicate the level of fluid in the bottle. The other bottles were completely filled with the formolized toxin and the bottles capped. Both sets of bottles were incubated as described above. It was found

that those bottles completely filled with the mixture would decolorize methylene blue, whereas the others did not. The toxoids from this experiment indicated that there is a slight but definite advantage to be gained by toxoiding anaërobically; the advantage is just definitely detectable using the flocculation technique.

Routine procedure.

These data have been employed in routine work. With toxins with an amino-nitrogen content of about 0·5 N/10 the reaction is adjusted to pH 8·4 with NaOH, and then the formalin added; the reaction immediately falls to about pH 7·6, and continues to fall more slowly until it reaches pH 6·7 in two days of room-temperature. The reaction is then adjusted to pH 7·5 with NaOH, and the mixtures transferred to 30° C. for four weeks. The loss of antigen is scarcely detectable. If the flocculation technique is considered accurate to 5 per cent, then the losses are within the limits of accuracy of the method of assay.

Summary.

(1) When semi-synthetic media diphtheria toxins are toxoided, the following five variables affect the quality of the toxoid: (*a*) reaction, (*b*) temperature, (*c*) formalin concentration, (*d*) amino-nitrogen content of the toxin, (*e*) redox potential.

(2) Evidence is given to indicate a *critical temperature*, 30–32° C. for diphtheria toxin, at pH 7·6.

(3) The significance of this critical temperature is shown.

(4) Data are given from which the amount of formalin to use with such toxins may be found.

(5) A method is described for the conversion of diphtheria toxin, in semi-synthetic medium, to toxoid, with not more than a 5 per cent loss of antigen, and with a small increase in Kf.

PART C.—THE LARGE SCALE PURIFICATION AND CONCENTRATION OF DIPHTHERIA TOXOID.

Crude toxoids prepared by the methods described contain, in addition to the specific toxoid, a variety of impurities. The composition of such toxoids may roughly be classified as follows: (1) Residual unutilized amino-acids, and perhaps some maltose; (2) mineral salts; (3) protein—(*a*) the specific antigen, (*b*) other bacterial protein; (4) pigments, of which there are at least three in appreciable amounts: (*a*) a yellow fluorescent flavine-like pigment, (*b*) a red pigment (largely coproporphyrin III), (*c*) a black or dark brown pigment.

(1) *Removal of the red pigment, phosphates and some non-toxoid protein.*

Unless removed before alum precipitation of the toxoid, the red pigment gives to the A.P.T. a pink coloration. It is desirable, therefore, that the coloured impurities be removed. After testing many substances, including decolorizing charcoals, to remove selectively the red pigment from crude toxoid, two gels or creams were found to be of value. The first, aluminium benzoate, did remove much of the red pigment, but for a rather serious loss of antigen.

The second, magnesium hydroxide (made by adding to a dilute solution of NaOH slightly less than its equivalent of magnesium sulphate, allowing the suspension to stand one week and discarding the supernatant), *in small amounts*, absorbed nearly all the pigment for 5–10 per cent loss of toxoid. The toxoid to be processed is adjusted to pH 8·5 before the cream is added. The following result is from a typical trial run on crude toxoid:

The reaction of a quantity of toxoid was adjusted to pH 8·5 with NaOH, and to 40 ml. samples were added increasing amounts of the $Mg(OH)_2$ gel ($\pm$ 0·4 M). These were allowed to stand undisturbed for 30 minutes, the supernatants decanted off, neutralized, and their titres determined by the flocculation technique; their colours were also observed:

	Vol. $Mg(OH)_2$ cream added.	% $Mg(OH)_2$ gel used.	Supernatant. Lf.	Kf.	Colour.	Antigen loss %.
1	*nil* (control)	*nil*	60	20	Orange	—
2	0·8 ml.	2	57	23	Pale orange	3
3	1·2 ml.	3	57	24	Yellow	2
4	1·6 ml.	4	55	25	Pure yellow	5
5	2·0 ml.	5	50	25	do.	12
6	2·4 ml.	6	47	26	do.	16

It was for the removal of the red pigment only that this reagent was first investigated. Subsequently the findings of Parventjev, Waldschmidt and Wiel (1942) were published. These workers were interested in the removal of unwanted *protein*, and do not appear to have noticed or encountered the pigment-removing properties of this hydroxide. The above samples were analysed for total protein. Usually for a 15 per cent loss of antigen the purity of the toxoid was increased from 600 Lf/mg. protein nitrogen to 800–1000. These results confirm the findings of Parventjev *et al.* that $Mg(OH)_2$ cream selectively adsorbs non-toxoid protein from diphtheria toxoid. Powdered MgO and $Mg(OH)_2$, but not $MgCO_3$, behave similarly, but have not the same degree of selectivity obtained with $Mg(OH)_2$ gel.

It was found that when toxoid derived from the modified Mueller's medium was partially purified with $Mg(OH)_2$ gel much of the inorganic phosphate was precipitated. The substitution of ammonia for NaOH, however, before adding the $Mg(OH)_2$ to the toxoid resulted in the almost complete removal of the phosphates, as magnesium ammonium phosphate, without interfering with the adsorption of the red pigment. The treatment of crude toxoid with $Mg(OH)_2$, therefore, serves as an excellent method of removing not only the red pigment, much unwanted protein, but also the inorganic phosphates, and gives a partially refined formol-toxoid eminently suitable for further processing.

(2) *Concentration of the toxoid using cadmium chloride.*

A variety of methods were tried for concentrating the toxoid present in the $Mg(OH)_2$ filtrate. These included adsorption on to calcium phosphate, zinc phosphate, and aluminium hydroxide, but all required relatively large amounts of each reagent, and gave only small volume concentrations. It was then found that cadmium chloride was a much better reagent, and gave a volume con-

centration factor of about 500-fold in one step. The addition of $Mg(OH)_2$ to crude formol-toxoid, as we have seen, results in the precipitation of the phosphates, and it is these which presented a major difficulty to earlier workers who used cadmium chloride to precipitate toxoid.

Preliminary experiments showed that small quantities of cadmium chloride would precipitate all the antigen present in $Mg(OH)_2$ filtrates, giving a slow forming precipitate of small volume. The amount of cadmium required depends on the reaction; with increasing acidity the amount increases, while the rate of precipitation and the volume of precipitate diminish. Also the colour of the precipitate is darker brown the more alkaline the mixture. These points are illustrated in the following experiments:

To 50 ml. quantities of a neutralized $Mg(OH)_2$ filtrate were added increasing amounts of a 50 per cent w/v $CdCl_2$ $2H_2O$ solution, and the mixtures adjusted (1) to pH 5·0 and (2) to pH 6·0, and allowed to stand at room temperature for 24 hours. These were then centrifuged and the supernatants assayed for toxoid content, using the blending technique of Glenny *et al.* (1926). The precipitates were dissolved in 2 ml. of 10 per cent Na citrate, and the volume made up to 50 ml. and then assayed for antigen content. The results of this experiment are shown in Table VII.

TABLE VII.—*Determination of the Antigen Precipitating Power of* $CdCl_2$ $2H_2O$ *at pH 5·0 and pH 6·0, using a* $Mg(OH)_2$*-treated Formol-Toxoid Filtrate.*

Final conc. of $CdCl_2$ $2H_2O$.	Series 1 pH 5·0.			Series 2 pH 6·0.		
	Supernatant.	Precipitate (→50 ml.).		Supernatant.	Precipitate (→50 ml.).	
% w/v.	Titre. (Lf.)	Titre. (Lf.)	Recovery. (%.)	Titre. (Lf.)	Titre. (Lf.)	Recovery. (%.)
0·2	37	2	5	32	10	25
0·4	30	10	25	10	30	75
0·6	27	17	42	0	40	100
0·8	17	22	55	0	40	100
1·0	15	25	62	0	40	100
1·25	10–12	27	67	0	40	100
1·5	10	30	75	0	40	100

An examination of a sodium citrate solution of the cadmium precipitated toxoid revealed that, although by the flocculation technique it appeared unaltered and recovered in good yield, it was present as a metallo-complex, in that its pH solubility characteristics and precipitation by ammonium sulphate were quite different from "normal" toxoid. It was then found that by dissolving the centrifuged cadmium-toxoid complex in a 5 per cent solution of $Na_2HPO_4 12H_2O$ to give a titre of about Lf 2500, and adjusting the reaction to pH 8·4 with NaOH, that all the cadmium present was precipitated as the phosphate, leaving all the toxoid in solution. Complete precipitation of the cadmium, however, requires 2–3 days, but may be accelerated by the addition of 2–5 per cent w/v of sodium chloride. The concentrated solution of toxoid may be analysed for freedom from cadmium, or alternately to a sample adjusted to Lf 1500 is added half its volume of a saturated solution of ammonium sulphate, when not more than 10 per cent of the toxoid should be precipitated.

(3) *Fractionation with ammonium sulphate.*

The concentrated cadmium-free solution of toxoid is diluted to Lf 1500, and half its volume of a saturated solution of ammonium sulphate added. The mixture (pH 7–7·2) is left for one hour for the precipitate to flocculate out, and then centrifuged. To the supernatant is then added crystalline ammonium sulphate to give a total concentration of 66 per cent saturation. The mixture is again left one hour and then filtered. The precipitate and filter-paper are together placed in a cellophane dialyzing bag (grade 400) and dialyzed against distilled water for 10 days in the refrigerator, with daily changes of water.

The following is a tabulated account of the details made during the processing of a 64 litre batch of crude toxoid :

Exp. 2441. 19/iii/45.

Stage.	Volume. (Litres.)	Titre. (Lf.)	Purity. (Lf/mg. $P.N_2$.)	Total quantity. (Megaunits.)	Total recovery. (%.)
(1) Original material	64	62	700	3·8	—
(2) $Mg(OH)_2$ filtrate	67	50	840	3·3	87
(3) Decomposed Cd. toxoid ppt.	2·0	1500	1000	3·0	80
(4) S33–S66 ppt. (dialyzed)	0·87	3000	1400	2·6	68

The dialyzed concentrated solution of toxoid, which contained 0·13 per cent ammonium sulphate, was freeze-dried, when 0·275 megaunit was found to weigh 1·4 g., or 196 Lf/mg. Since pure toxoid is probably 340 Lf/mg., this sample of purified toxoid is 58 per cent pure by weight, and 65 per cent by protein content.

Summary.

Toxoid derived from toxin produced in a dilute semi-synthetic medium is purified and concentrated in three stages :

(1) Preliminary treatment with $Mg(OH)_2$ to remove the red pigment, the inorganic phosphates and some unwanted protein.

(2) Concentration and elimination of non-protein substances using cadmium chloride.

(3) Fractionation of the concentrated toxoid with ammonium sulphate.

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THE IMMUNOLOGICAL RELATIONSHIPS OF STRAINS OF ALASTRIM VIRUS.

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IN an earlier paper (Horgan and Haseeb, 1939) on cross-immunity experiments in monkeys between variola, alastrim and vaccinia viruses, the existing evidence on the antigenic constitution of alastrim was discussed. It was concluded "that strains of varying antigenic constitution are included under the general name alastrim, and there seems to be some evidence that such differences may be related to geographical distribution." A review of the literature also indicated that differences of infectivity for animals existed between different strains. It was concluded that "the evidence from experimental animals is, however, far too scanty to enable any general conclusions concerning the antigenic identity or relationships of various alastrims to be drawn, and for the present it would perhaps be wiser to regard results obtained from a strain of alastrim responsible for a particular outbreak, or epidemic, as being strictly applicable to that strain only, and to be used with caution when dealing with a strain of different provenance.

"Some of the difficulties (at least) in discussions on alastrim seem to arise from the assumption, perhaps an unconscious one, that the very uniform epidemiological and clinical features of outbreaks of alastrim throughout the world are necessarily associated with antigenic identity of the infecting strains. The position may not be unlike that of the Salmonella group, where enteric fevers, often with an identical clinical picture, are due to Salmonella strains antigenically related but with marked specific differences.

"It is obvious that, before the problem is finally solved, a direct antigenic comparison of different strains of alastrim in experimental animals will be required."

Further researches were stopped by the war, and it was not until 1945 that the authors found an opportunity to resume work on the problem. Infective material mostly in the form of scabs was received from various African outbreaks of alastrim, and a series of cross-protection tests in monkeys was carried out. The work could not be completed owing to the difficulty of obtaining takes with some of the strains, and the absence of fresh infective material delayed further work until 1947. In July 1947 an outbreak of mild alastrim occurred in Equatoria Province, Southern Sudan, and thanks to the help of the Senior Medical Inspector, Dr. R. B. Usher Somers, Juba, infective material—pus, moist scabs and dry scabs—was sent packed in ice in vacuum flasks to Khartoum. It was hoped to carry out cross-protection tests between the Juba and the other African strains, but unfortunately the latter material, although desiccated and stored *in vacuo* in the freezing chamber of a refrigerator, proved completely

inactive when rubbed on to the scarified bellies of monkeys. Curiously enough, material of the St. Louis strain used in the 1939 experiments and which had been stored under identical conditions gave excellent takes after eight years.

EXPERIMENTAL.

Full details of the preparation of suspensions of infective material and the technique of inoculation will be found in the earlier paper (Horgan and Haseeb, 1939). The method of testing for immunity is also the same except that undiluted virus suspensions were used.

Animals used and their susceptibilities.

Monkeys (*Cercopithecus aethiops aethiops* L.—referred to as *C. sebaeus* in earlier papers), rabbits—a pure bred albino strain highly susceptible to vaccinia virus—and white mice. The latter are a pure bred albino strain (Swiss) originally provided by the Yellow Fever Research Institute, Entebbe, and used extensively in yellow fever work. Positive results of varying degrees were obtained only in the monkeys, the rabbits and mice remaining completely refractive. These results are in accordance with previous experience in Khartoum and with those of the majority of workers. While this work was in progress Van Breuseghem's (1942) paper came to hand. This author, working in the Belgian Congo in 1940, succeeded in infecting cutaneously rabbits and white mice with a locally isolated strain of alastrim, a result in striking contrast to the earlier experiments of Van Hoof (1925). The latter completely failed to infect rabbits and goats and had the greatest difficulty in infecting monkeys (*Cercopithecus* sp ?), most of the latter animals also proving refractory.

On the contrary, Van Breuseghem succeeded after a single rabbit passage with human material in carrying out several serial passages in mice.

In view of these successful results, renewed attempts were made to infect rabbits and mice with human material. The rabbits were inoculated both cutaneously, intra-cutaneously, and intra-nasally; as in previous experiments with vaccinia virus the last route was found to be very successful in immunization, but all attempts completely failed. Mice were scarified on the base of the tail following Van Breuseghem's technique, but although yellowish crusts were usually present on the 3rd or 4th day it is certain that they were merely due to a traumatic reaction, as none of the mice when tested with vaccinia virus exhibited the slightest degree of immunity.

Strains of virus.

The word " strain " is used here without prejudice to its antigenic constitution, and merely indicates infective material from different outbreaks of alastrim, Strains were received from—

(*a*) Astrida, Territoire du Ruanda-Urundi, Eastern Congo (January and February, 1945).

The three Congo strains from typical mild clinical cases showed a very low degree of infectivity for the Sudan monkeys, some of the animals appearing to be completely resistant. Even in those which were infected the takes were poor, consisting of a few isolated small papules and pustules. The original failures may have been possibly due to inactivation of the virus in the scabs,

although all material was sent by air mail. But results were even less successful when attempts at serial monkey passage were made using freshly ground-up material from the papules on Monkey 1. Only one monkey, No. 1 (A) out of four inoculated gave a few scattered papules. Attempts at further passage (two monkeys) failed completely.

(*b*) Cape Colony—referred to hereafter as South African—from a mild clinical case.

(*c*) Mwanza district, Tanganyika.

Scabs and pus were received (March, 1945) from two very severe clinical cases from the widespread epidemic of 1944–45. This epidemic showed great variations in virulence in different areas, and in one area it was reported to have shown an enhancement in virulence with a mortality comparable to classical smallpox. The authors have not seen the completed official returns, but an analysis of cases reported in the Epidemiological Information Bulletin of 1945 and 1946 gave a total of 2789 cases with 334 deaths, i.e. a mortality of 12 per cent for the period from August 26 to October 27, 1945.

(*d*) Dar es Salam, Tanganyika, from a mild case during a very mild outbreak (March, 1945).

(*e*) St. Louis, U.S.A., originally supplied in 1939 by Dr. Markham, Ohio State University, and used in previous immunity tests (Horgan and Haseeb, 1939).

(*f*) Juba, Equatoria Province, Sudan (1947). These strains are of considerable interest as it is the first time alastrim virus has been isolated in the Sudan. Previous attempts (Horgan and Haseeb, 1939) were uniform failures, and no reason can be given why success was achieved in this case.

Alastrim and vaccinia viruses.

Although the present paper is not concerned specifically with this problem, the opportunity was taken to test with vaccinia virus some of the monkeys in Table I one month after immunity tests with the alastrim strains. The standard vaccine lymph as issued for human vaccination was used as the test strain.

Those tested were 1, 1(A), 2, 3, 4, 4 (A), 4 (B), 5, 8, 9 and 10; all showed similar semi-confluent takes of tiny abortive vesicles which had dried up completely by the 6th day (vaccinoid reactions). These results are in conformity with the earlier experiments, which have been fully discussed (Horgan and Haseeb 1939), and indicate that strains of alastrim can produce high but not complete immunity to vaccinia virus.

DISCUSSION.

The results in Tables I and II will first be discussed separately before a final assessment is made. The two Congo strains (1) (1*a*) and (2) give complete protection against South Africa (4*b*) and partial against Mwanza (1) and St. Louis strains in monkeys 1 and 1 (A), complete against South Africa (4*b*) and Mwanza (1) and partial against St. Louis strain in monkey 2. In monkey 3 it is obvious that the single papule produced by the immunizing strain Congo 3 was associated with no detectable immunity. The South Africa strain protected completely against itself in monkeys 4, 4 (A) and 4 (B) and partially against Mwanza (1) and St. Louis strains in monkeys 4 and 4 (A), but completely in monkey 4 (B). The Dar-es-Salam strain gave complete protection against the

TABLE I.—*Cross-Immunity Experiments on Monkeys between Different Strains of Alastrim, January–May, 1945.*

Monkey No.	Immunizing strain.	Result.	Days after immunization.	Test strains. South Africa (4b).	Tanganyika (Mwanza 1).	St. Louis.	Comments.
1	Congo (1)	Few isolated papules	77	0	±	±	Some of the papules scraped for material (1a).
1 (A).	,, (1a)	,, ,, ,,	72	0	±	±	..
2	,, (2)	,, ,, ,,	54	0	0	±	..
3	,, (3)	One papule	37	+	+	+	..
4	South Africa	Confluent take	67	0	±	±	Some lesions scraped on 4th day to provide material (4a).
4 (A).	,, ,, (4a)	,, ,,	62	0	±	±	Ditto (4 b).
4 (B).	,, ,, (4b)	,, ,,	23	0	0	0	..
5	Tanganyika, Dar-es-Salam	,, ,,	57	0	±	±	..
6	Tanganyika, Mwanza (1)	,, ,,	39	0	0	0	..
7	Tanganyika, Mwanza (2)	,, ,,	34	0	0	±	..
8	—	—	—	++	—	—	Control.
9	—	—	—	—	++	—	,,
10	—	—	—	—	—	++	,,

TABLE II.—*Cross-Immunity Tests, June–July, 1947.*

Monkey No.	Immunizing strain.	Result.	Days after immunization.	Test strains. Juba. 3.	Juba. 3 (A).	St. Louis.	Comments.
11	Juba (1)	?	67	+	+	+	Doubtful take. A few small pustules which resembled impetigo.
12	,, (1)	?	67	+	+	+	Ditto, ditto.
13	,, (2)	Confluent take	57	+	+	+	In spite of good primary take no immunity could be demonstrated. Some of the lesions scraped to provide fresh material (2 A).
14	,, (3)	,, ,,	50	0	0	±	Lesions scraped on 4th day to provide fresh material (3 A).
15	,, (2*a*)	Negative	—		—	—	Died of T.B. before immunity test carried out.
16	,, (3*a*)	Confluent take	43	0	0	±	,,
17	St. Louis	,, ,,	41	0	0	0	,,
18	,,	,, ,,	41	0	0	0	,,
19	,,	,, ,,	—		—	—	Killed on 4th day and lesions scraped to provide fresh material.
20	—	—	—	++	++	++	Control.
21	—	—	—	++	++	++	,,
22	—	—	—	++	++	++	,,

Interpretation of Symbols.

++ = confluent reaction (primary take).
± = semi-confluent or scattered pustules drying to scabs on or before sixth day.
± = a few abortive pustules (less than 10) drying up before sixth day.
0 = absence of reaction.
— = not done.

The figures in brackets after the immunizing strains, e.g. Congo (1), indicate that the original infective material, pus or scabs, came from different cases of human alastrim in various outbreaks. Small letters after the figures, e.g. Juba (2*a*), indicate material from first monkey passage of respective strain (Juba 2).

South Africa and partial against Mwanza (1) and St. Louis strains, while Mwanza (1) protected completely against all three test strains. Mwanza (2) protected completely against South Africa (4*b*) and Mwanza (1) and partial against St. Louis strain in monkey 7. A point of possible criticism is that the intervals between immunizing and testing varied considerably, ranging between 23 and 77 days, and it is therefore possible that after the longer intervals immunity was waning. While theoretically this possibility may be admitted, there appears to be little correlation between the interval and subsequent immunity elicited by the tests. Thus in all monkeys, excluding the failure No. 3, immunity was complete against the South Africa and strong against Mwanza (1) and St. Louis strains. It is, of course, possible that although the three controls reacted with confluent takes, the potency of the South Africa strain may have been lower than that of Mwanza (1) and St. Louis strains. Shortage of monkeys, however, rendered it impossible to titrate out each test strain to end point. It should, however, be emphasized that all immunity tests, together with the three controls, were carried out on the same day with the same virus suspensions in each case, in order to eliminate possible variations in potencies between suspensions of the virus prepared at different times. As the monkeys were infected at different dates according to the availability of infective human material, there were inevitably differences in the intervals between immunizing and immunity testing.

In Table II it appears that no active virus was present in the human material from Juba (1), monkeys 11 and 12. It is difficult to explain the absence of immunity in monkey 13, in spite of a confluent take by the immunizing material, Juba (2). The most reasonable explanation is the loss of immunity during the 57 days' interval, but it is quite possible that, as in the case of duration of immunity in man after vaccination, there are also considerable individual variations in immunity following alastrim infection in monkeys. As far as the authors are aware there are no records in the literature of the average period of immunity after alastrim (in monkeys).

In monkeys 14 and 16, Juba (2) and (3) gave complete protection against Juba (3) but partial against St. Louis strain, while the latter protected completely against itself and Juba (3) in monkeys 17 and 18. The three controls gave confluent reactions against the test strains. Summing up the combined results, it is a reasonable inference that while strains of alastrim of different provenance are closely immunologically related, they are not identical. Some strains, e.g. St. Louis and Mwanza, possess a higher content of the basic immunizing antigen, and in this respect might be termed "master-strains." These results are closely paralleled by the immunological relationships of strains of the animal pox virus (or group of viruses) affecting sheep and goats, known as contagious pustular dermatitis (Horgan and Haseeb, 1947). There is as yet insufficient evidence to make any dogmatic pronouncement whether these differences are of a qualitative or a quantitative character, or both, but the authors consider that the results on the whole support the quantitative hypothesis; in other words, that all strains possess a common antigenic complex, the factors of which vary quantitatively. The alternative qualitative hypothesis, namely that certain strains in addition to the basic complex possess other minor antigens, cannot, however, be excluded. This matter has been more fully discussed in the authors' earlier paper on cross-immunity tests between variola, alastrim, and vaccinia (Horgan and Haseeb, 1939). The conclusion there reached in favour

of quantitative differences was criticized on the grounds of " the different degree of susceptibility shown by the monkey to the viruses, vaccinia virus having a much higher degree of infectivity for this animal than either variola or alastrim. . . . Even with a comparable infecting dose the virus with the greatest infectivity is going to out-multiply the others and so provide a greater immunizing dose and antigenic stimulus " (Editorial, 1940). The susceptibility of monkeys to alastrim virus will be discussed below, but in the present series, in those animals which show an equally good (confluent) response to the immunizing strains, it is a reasonable inference that the susceptibility and consequently the antigenic stimulus is of the same order. The results, however, definitely show that certain strains produce a higher immunity, and the authors still consider that the results can be best explained by postulating slight quantitative variations in the immunogenic complex.

Susceptibility of monkeys to alastrim strains.

On the other hand, the varying susceptibility of monkeys (*Cercopithecus* spp.) and other animals such as rabbits or mice to alastrim strains of different provenance and at various times suggest qualitative differences in those strains. It was shown that the same monkey completely refractory to Khartoum alastrim was fully susceptible to a St. Louis strain (Horgan and Haseeb, 1939), and in the present series the failure to establish a Congo strain has been noted above. One cannot, of course, exclude variations in the susceptibility of individual monkeys, but the evidence as a whole strongly suggests that differences in the strains of alastrim play a more important role. The existing literature has been reviewed (Horgan and Haseeb, 1939), and with the exceptions of Van Breuseghem's results noted above, no further work has apparently been carried out on this problem, judging at least by the absence of any references in the literature to date. But these qualitative differences in the infectivity of different strains towards monkeys are in no way inconsistent with the hypothesis of quantitative immunogenic differences, unless one assumes that the same antigenic complex is responsible for both the infectivity of a strain and its immunogenic capacity.

Human pathogenicity and immunogenic relationships.

While it was unfortunately impossible to carry out the complete series of cross-immunity tests, the results do not suggest that any close relationship exists. The South African and Mwanza (2) strains completely cross-protected in monkeys 4 (B) and 7, but the former was derived from a very mild case in a mild outbreak, whereas the latter, as already noted, was derived from severely ill patients in a great epidemic of considerable virulence. It is interesting to note that in the earlier cross-protection experiments (Horgan and Haseeb, 1939) between variola and alastrim (St. Louis) viruses the latter was the more efficient immunizing agent. The suggestion was then made " that the factor which confers virulence on the complex variola antigen is not associated with immunogenic capacity," and a similar inference can be drawn from the present experiments. Again, there is no evidence that any relationship exists between the pathogenicity (for man) of strains of variola or alastrim viruses and their infectivity towards monkeys.

The differing degrees of infectivity of alastrim strains for monkeys could be most simply explained by a similar hypothesis, as for variola in man, namely,

that infectivity is due to specific antigens different from the basic immunogenic complex (vaccinia). This suggestion has been criticized (Editorial, 1940), "it is difficult to believe that specific antigen of smallpox virus, which is responsible for its virulence for man, should play no part in the immunity to smallpox, and it seems unlikely that degradation of the virus should make it more infective for any animal species." It has, however, been conclusively proved (Horgan and Haseeb, 1945) that strains of vaccinia irrespective of their origin completely cross-protect, and during the war experience of human vaccination on a large scale showed beyond doubt that the vaccine lymph used in these Laboratories and originally derived from a strain of mild English alastrim was capable of giving complete protection against the virulent smallpox rife in the Sudan and the Middle East.

There is also a misunderstanding about the use of the word "degradation." The authors used it merely to imply loss of a particular antigen or antigens, but not in the sense of loss of virulence. The analogy of the Salmonella group may be quoted where antigenic degradation, i.e. loss of antigens, is associated with a high virulence and specificity, e.g. in the fowl Salmonellas or in *Bact. typhosum.*

The above discussion can be briefly summarized as follows: Strains of alastrim virus have a basic immunogenic complex (vaccinia) which has minor quantitative variations. There are, however, marked differences in their pathogenicity for man and infectivity in monkeys, and such differences are likely to be associated with specific antigenic factors.

The conclusion is drawn that the clinical syndrome of alastrim in man is caused by a group of closely related viruses rather than a single strain, and from the point of view of virus nomenclature it would be more correct to speak of the "alastrim group" or alastrim viruses than the current term in the singular, "alastrim virus."

The present paper follows the universal usage of "variola virus" in the singular, but experimental evidence is required to confirm or disprove this assumption, for it is possible that epidemics of classical or virulent smallpox may likewise be caused by closely related strains of the virus.

SUMMARY.

1. Cross-protection tests between several strains of alastrim from different sources in Africa and the U.S.A. indicate that while immunity is not fully reciprocal, the strains are closely related immunogenically.

2. The varying susceptibility of monkeys (*C. aethiops aethiops* L.) to different strains is also discussed, and it is concluded that the results are best explained by the theory of minor qualitative antigenic differences in the strains.

3. The present results are in accordance with the earlier work of the authors, namely, that (human) pathogenicity of strains of alastrims or variola is unrelated to their immunogenic capacity in experimental monkeys.

4. Strains of alastrim produce strong but not complete immunity to vaccinia virus and it is suggested that all possess a common immunogenic complex with minor quantitative variations.

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